REMARKS

Entry of this Amendment is proper under 37 C.F.R. 1.116, because the Amendment places the application in condition for allowance for the reasons discussed herein; does not raise any new issue requiring further search and/or consideration, and places the application in better form for an appeal should an appeal be necessary.

Claims 150-152 have been canceled without prejudice or disclaimer to the subject matter recited therein. Applicant reserves the right to file one or more continuation applications(s) directed to any of the canceled subject matter.

Claims 1, 13, and 22 have been amended and new claims 153-155 have been added. In particular, claim 1 has been amended to delete the phrase "isolated," to recite "consisting of" with regard to the recited fragments, and to note that the peptide is a collagen type I-binding peptide. Claim 13 has been amended to remove the phrases "isolated" and "fragment". Claim 22 is amended to recite "consisting of" with regard to the amino acid sequence of the cytoplasmic domain. New claims 153-155 have been added and relate to the integrin subunit α11 and fragments of same. Support for this amendment and the new claims can be found throughout the specification and claims as-filed, at least at originally filed claims 12-14. No new matter has been added by any of the present amendments and new claims to the subject application.

Applicants request that the Office affirmatively acknowledge that the Replacement Sheets for Figures 1-8 are accepted, as submitted on May 21, 2005, are accepted.

Claims 1, 13, 22 and 150-152 have been rejected under 35 U.S.C. § 112, first paragraph, for purportedly not being enabled for the full scope of the claims. As claims 150-152 are canceled herein, Applicants address the present rejection as applying to claims 1, 13, 22 and new claims 153-155.

First, Applicants submit that the claims as amended herein, are directed to the fragments of amino acid sequences from amino acid 1165 to amino acid 1188 of SEQ ID No. 2 and from amino acid 804 to amino acid 826 of SEQ ID No. 2. Applicants note with appreciation that the Office agrees that these fragments are enabled.

With regard to the fragments as *"comprising"* the amino acid sequence from amino acid 159 to amino acid 355 of SEQ ID No. 2 (*i.e.* the I-domain of the integrin alpha-11 subunit), Applicants submit that they are enabled by the specification. To this end, Applicants submit that at the time the present application was filed (and even at the time the first priority application was filed) the skilled artisan could readily make the fragment as claims and then test it for collagen type I-binding activity without undue experimentation.

For example, the specification provides detailed comments on testing a peptide fragment for collagen-binding activity by chromatographic means (*see* page 18, line 18 to page 19, line 2 and page 26, line 12 to page 27, line 8).

The Office specifically notes that undue amount of experimentation would be required to arrive at the breadth of fragments of the integrin alpha-11 subunit which bind collagen type I since the relationship between the peptide and its activity was not well understood. Applicants respectfully disagree.

The involvement of the I-domain in the collagen-binding activity of integrin a subunits was known at the priority date of the present application. The role of the I-

domain in collagen binding is well recognized, and is discussed in the introduction of the present specification on page 2, lines 6 to 25. Thus, the application as-filed provides clear guidance as to the amino acid sequence requirements to retain collagen-binding activity.

As further support that the role of the I-domain of integrin a subunits in collagen binding was well understood at the time the present application was filed, Applicants enclose a review article by Dickeson & Santoro (*see*, for example, the paragraph bridging pages 562 and 563). This reference was published in 1998, one year before the priority date of the application as-filed

The Office further states that experimental techniques for testing ligand binding properties of cell surface receptors were not in routine use at the priority date of the application as filed. Applicants respectfully disagree. The chromatographic method described on page 18, line 18 to page 19 of the present specification, for testing a peptide fragment for collagen-binding activity, was first published in 1990 (see Gullberg *et al.*, 1990, *Exp. Cell Res.* 190:254-264, copy enclosed for the Office's convenience). This reference shows that suitable methods for determining the collagen binding activity of integrin peptides were available nine years before the priority date of the present application.

By way of further support, Applicants provide copies of several references located on the PubMed online database, relating to collagen-binding activity of integrin I-domains. Significantly, several of these references describe testing collagen-binding activity of fragments of integrin a subunits comprising the 1-domain. Enclosed for the Office's review in this regard are: Calderwood et al., 1997, *Biol. Chem.* 272:12311-12317; Dickeson et al., 1997, *Biol. Chem.* 272:7661-7668;

Kamata & Takada, 1994, *Biol. Chem.* 269:26006-26010; Kern *et al.*, 1994, *Biol. Chem.* 269:268 1 1-228 16; and Tuckwell et al., *1995,3?. Cell Sci.* 108:1629-1637.

As shown by these references, methods of testing the collagen-binding activity of peptides comprising the I-domain of an integrin alpha subunit were well known and routinely used prior to the filing of the present application. Thus, the skilled artisan could readily make and test the collagen-binding activity of peptide fragments comprising the I-domain of the integrin a subunit of the present invention without undue experimentation.

Applicants further submit that the specification provides sufficient enablement for fragments comprising the peptides as specified in claims 1 and 22 (as amended herein). Applicants submit that a skilled artisan could make the claimed fragments comprising such peptides and test these fragments for the desired properties without undue experimentation. The specification provides guidance as to how to test a peptide fragment for collagen-binding activity by chromatographic means (*see*, page 26, line 12 to page 27, line 8). The Office asserts that altering the amino acid sequence of a polypeptide can alter its properties. However, even if this is the case, Applicants submit that testing a given fragment of the integrin subunit sequence of SEQ ID No. 2 to determine whether it retains the desired collagen-binding activity could be accomplished without undue experimentation.

In view of the above, the Examiner is respectfully requested to withdraw each and every one of the enablement rejections.

Additionally, claims 1, 11, 13, 14, 20, 22-25, 28, 94, 113, 115, 116, 119, 121-124, 126, 146, and 149 have been rejected under 35 U.S.C. § 112, first paragraph,

for purportedly not satisfying the written description requirement. This rejection is respectfully traversed.

The Office appears to argue that the applicant was only in possession of the intact integrin alpha-11 subunit and the fragments thereof exemplified in the application as filed. However, Applicants submit that it is not required to exemplify every embodiment of the invention in the specification. The objective standard for determining compliance with the written description requirement is, "does the description clearly allow persons of ordinary skill in the art to recognize that he or she invented what is claimed." *In re Gosteli*, 872 F.2d 1008, 1012, 10 U.S.P.Q.2d 1614, 1618 (Fed. Cir. 1989).

To this end, Applicants submit that they were in possession of the integrin alpha-11 subunit and fragments encompassed by the claims as amended herein. Applicants note with appreciation that the Office acknowledges that the intact integrin alpha-11 subunit and fragments consisting of the amino acid sequences from amino acid 1165 to amino acid 1188 of SEQ ID No. 2 and the amino acid 804 to amino acid 826 of SEQ ID No. 2 are supported by the specification.

Applicants further submit that the specification provides adequate support for the recitation of fragments "comprising" the amino acid sequence from amino acid 159 to amino acid 355 of SEQ ID No. 2 (the I-domain of the integrin all subunit). As discussed above, *supra*, the knowledge of the art at the time the application was filed combined with the present specification provides support for the fragments. To this end, the specification provides detailed comments on testing a peptide fragment for collagen-binding activity by chromatographic means (*see* page 18, line 18 to page 19, line 2 and page 26, line 12 to page 27, line 8).

In light of the above, withdrawal of this written description rejection is respectfully requested.

Claims 1, 13, 151-152 have been rejected under 35 U.S.C. § 102(b) as supposedly anticipated by Gullberg et al. (Dev. Dyn., 204:57-65 (1995)) ("Gullberg"), as evidence by Velling et al. ("Velling"). This rejection is respectfully traversed. As discussed above, claims 151-152 have been canceled without prejudice or disclaimer. Therefore, the Examiner's rejection is moot as to those claims, and Applicants address the present rejection as to claims 1, 13 and 153-155.

Applicants note that the present claims are amended herein to recite only a recombinant integrin all subunit. Gullberg does not disclose a recombinant integrin subunit.

The Office asserts that the 'alpha-mt' subunit of the cited reference is identical to the alpha-11 subunit of the present invention. However, the Office fails to provide evidence to support this assertion. As the Office is aware, the fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic. *In re Rijckaert*, 9 F.3d 1531, 1534, 28 U.S.P.Q.2d 1955, 1957 (Fed. Cir. 1993) (emphasis added); *In re Oelrich*, 666 F.2d 578, 581-82, 212 U.S.P.Q. 323, 326 (CCPA 1981). "In relying upon the theory of inherency, the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art." *Ex parte Levy,* 17 U.S.P.Q.2d 1461, 1464 (Bd. Pat. App. & Inter. 1990) (emphasis in original).

The Office refers to page 25, lines 32 to 37 of the application in support , wherein it is stated that the integrin alpha-11 subunit of the invention is "identical" to the integrin alpha-mt subunit disclosed in Gullberg. Applicants note that this passage of the specification discusses the similarity of the two integrin alpha subunits in terms of physical properties and *in vitro* expression, and states at page 25, lines 32-27, that:

> "Based on similar SDS-PAGE migration patterns, similar behaviour under reducing conditions, association with beta] integrin chain, and up-regulation during in vitro differentiation of human foetal myoblasts, the present data show that alpha-11 integrin is identical 14'ith alpha-mt."

Therefore, the comparison is *not* based on the nucleotide and amino acid sequences of the alpha-mt and alpha-11 integrin subunits. The basis for this comparison is important, because the integrin subunit of the present invention is defined with reference to a specific, full-length amino acid sequence (SEQ ID No. 2). The Office does not provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied art, and Applicants submit that it cannot be concluded with any degree of certainty that the alpha-mt subunit comprises the amino acid sequence of SEQ ID No. 2.

For prior art to be anticipatory, every element of the claimed invention must be disclosed, either explicitly or inherently, in a single item of prior art in the form literally defined in the claim. *See, e.g., Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 231 U.S.P.Q. 81, 90 (Fed. Cir. 1986). Therefore, in the present instance, a conclusion of lack of novelty should only be reached if it can be shown that the alpha-mt subunit disclosed in the Gullberg *et al.* paper comprises an identical sequence to that shown in SEQ ID No. 2 of the present application. The Office has failed to show this.

The amino acid sequence of the alpha-mt subunit is not known. In Gullberg, the alpha-mt subunit was identified in human foetal G6 myotubes by immuno-precipitation using antibodies to the beta-i integrin subunit. The study described in Gullberg did not extend to the cloning and sequencing of the alpha-mt subunit. In fact, the alpha-mt subunit from human foetal G6 myotubes has never been sequenced.

The authors of Gullberg, who include the present inventor, have attempted to clone the alpha-mt gene from human foetal G6 myotubes, but did not succeed. As noted in the present specification, "In order to determine the nature of the integrin chain that we had previously characterized on human fetal muscle cells and named alpha-mt (38) [reference 38 is the cited Gullberg reference], a number of approaches were used Applying PCR with mRNA from fetal muscle cells as template together with degenerate primers to conserved regions of integrin alpha subunits (43) we amplified cDNA for alpha-i, alpha-4, alp ha-5, alpha-6 and alpha-v integrin chains (data not shown), but failed to amplify the novel integrin." *See* page 19, lines 21 to 28 of the specification.

Only when the inventor of the present inventor took the inventive step of using a different cDNA library to that used in the Gullberg, namely a human *uterus* cDNA library, that he succeeded in cloning the alpha-11 gene (*see* page 19, line 37 to page 20, line 6 of the application as filed).

In addition, Applicants note that a different source material was used for cloning the alpha-II gene of the present invention from that of Gullberg. Thus, the alpha-mt subunit disclosed in Gullberg is not an identical sequence to that of the alpha-11 subunit the present application. For example, one of several different possibilities is that the alpha-11 and alpha-mt subunits are homologues or allelic

variants with one or more amino acid differences. Such homologues or allelic variants would not destroy the novelty of the subject matter of the pending claims because they would not comprise an amino acid sequence identical to that of SEQ ID No. 2.

The Office seems to assert that the amino acid sequence of SEQ ID No. 2 is an inherent property of the alpha-mt subunit disclosed in Gullberg. For support, the Office cites a paper which published after the present priority date by Velling. In response, Applicants enclose Declarations by Dr. Gullberg and Dr. Velling, providing their comments as to the Office's interpretation of the Gullberg and Velling references discussed in the outstanding Office Action.

Inventor Gullberg declares that it is not possible to conclude that the alpha-mt subunit disclosed in his earlier paper and the alpha-11 subunit of the present invention are identical. Similarly, Dr Veiling declares that it is not even probable that the alpha-mt subunit and the alpha-11 subunit share 100% sequence identity, because they are derived from different tissues.

In light of the above, Applicants request that the present rejection under 35 U.S.C. § 102 be withdrawn.

CONCLUSION

In view of the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order. Such action is earnestly solicited.

In the event that there are any questions relating to this Amendment and Reply, or the application in general, it would be appreciated if the Examiner would

telephone the undersigned attorney concerning such questions so that the prosecution of this application may be expedited.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

Date: November 21, 2005

By: _____
Deborahy H. Yellin
Registration No. 45,904

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Attachments: Declarations of Drs. Gullberg and Velling
Literature cited in Amendment

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of	)	Examiner: Maher Haddad
	)	
Donald Gullberg	)	Art Unit: 1644
	)	
Serial No. 09/980,403	)	
	)	
Filed: April 15, 2002	)	
	)	
For: "Integrin Heterodimer And An Alpha Subunit Thereof"	)	

DECLARATION OF PROFESSOR DONALD GULLBERG

I, Professor Donald Gullberg, hereby declare that:

1. I am a Professor of Biomedicine at the University of Bergen, Norway.

2. My *curriculum vitae* is provided as Appendix 1.

3. I am the sole inventor in respect of US Patent Application No. 09/980,403, which relates to the human integrin alpha-11 subunit and its use.

4. I have reviewed the examination report dated 19 May 2005 issued in connection with US 09/980,403, including the references cited therein.

5. I have been asked to comment on the following scientific paper, on which I am named as the first author:

   Gullberg *et al.* 1995, *Dev. Dynamics* **204**, 57-65

6. My paper describes the purification of a novel integrin alpha chain, which we designated alpha-mt, from human fetal myotubes.

7. The examiner asserts that the integrin alpha-mt subunit is identical to the alpha-11 subunit described in US 09/980,403. In support of this assertion, the examiner has cited the passage at page 25, lines 32 to 37 of US 09/980,403, wherein it

is stated that '*the present data show that alpha-11 integrin is identical with alpha-mt*'.

8. However, this conclusion is based on the physical properties of the alpha subunits and their *in vitro* expression, not on their amino acid sequence. This is important because the polypeptides claimed in US 09/980,403 are limited by reference to their amino acid sequence, namely SEQ ID NO: 2.

9. We have never been able to sequence the alpha-mt gene and determine the amino acid sequence of the encoded protein. In fact, to the best of my knowledge, the amino acid sequence of the alpha-mt subunit described in the Gullberg *et al.* paper has never been determined.

10. Hence, it is not possible to conclude that the alpha-11 subunit and the alpha-mt subunit are identical at the level of amino acid sequence.

11. Consequently, the examiner's assertion that the amino acid sequence of SEQ ID NO: 2 is an inherent feature of the alpha-mt subunit is lacking scientific credibility.

12. In fact, we attempted to clone the alpha-mt gene, as described in the Gullberg *et al.* paper, from human fetal G6 myotubes but did not succeed. It was only after we decided to use a different cDNA library to that used in the Gullberg *et al.* paper, namely a human *uterus* cDNA library, that we succeeded in cloning the alpha-11 gene.

13. Given that we used a different source material for cloning the alpha-11 gene, it is impossible to conclude that the alpha-mt subunit disclosed in the Gullberg *et al.* paper comprises an identical sequence to that of the alpha-11 subunit the present application. For example, one of several different possibilities is that the alpha-11 and alpha-mt subunits are homologues or allelic variants with one or more amino acid differences. However, such homologues or allelic variants would not comprise an amino acid sequence identical to that shown in SEQ ID NO: 2.

14. The Examiner supports his novelty objection with reference to a paper by Velling *et al.*, 1999, *J. Biol. Chem.* **274**:25735-25742. This paper, on which I am also named as an author, corresponds to the

disclosure in US 09/980,403 but was published after its filing date. Again, comparisons in this paper between the integrin alpha-mt subunit and the alpha-11 subunit described in US 09/980,403 are based on the physical properties of the alpha subunits and their *in vitro* expression, not on their amino acid sequence. Hence, it cannot be concluded that the alpha-mt subunit comprises the amino acid sequence of SEQ ID NO: 2.

15. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the above-referenced application or any patent issued thereon.

Professor Donald Gullberg

Nov. 10th, 2005
Date
Bergen, Norway

CURRICULUM VITAE

Name:	Donald Elon Gullberg
Date and place of birth:	1959-03-19 in Arboga, Sweden.
Norwegian Personal nr:	190359-19115
Marital status:	Married, two children born 1989 and 1992.
Home address:	Rieber Mohns vei 25A, 5231 Paradis, Norway.
University studies:	Major in chemistry, Uppsala university (1980-1984).
Degree:	Dr. Med. Sci. 90-02-21, Dept. of Medical Chemistry, Uppsala Univ.
Postdoctoral studies:	Swedish Natural Science Research council (NFR) postdoc scholarship (June 1990 - November 1992) UCLA, CA. Studies of cell adhesion during muscle development in *Drosophila melanogaster*.
Professor competence:	September 2003, qualified as professor in Medical Biochemistry, Karolinska institute (ranked number one for lectureship in Medical Biochemistry). February 2003, qualified as professor in Medical Physiology (ranked number one for professorship in Physiology at University of Bergen, Norway). November 2002, qualified as professor in Functional Genetics, Lund University (ranked number two for lectureship in functional Genetics). March 2002, qualified as professor in Medical Biochemistry at Swedish agricultural university (ranked number three for position as professor in Medical Chemistry).
Present position:	(040101 - present) Professor of Biomedicine, Dept. of Biomedicine, Division of Physiology, University of Bergen.
Previous positions:	**(990701- 031231)** Research scientist. During 990701-010830 at Dept. Cell & Molecular Biology, Uppsala University. From 01-09-01- 03-12-31at Medical Biochemistry and Microbiology, Uppsala University. **(990101-990630)** "Vikarierande lektor", Department of Cell-& Molecular Biology, Uppsala University. **(930101-981231)** "Forskarassistenttjänst" financed by Swedish Medical Research council (MFR), at Dept. of Animal Physiology
Prizes:	Walter A. Jonsson Prize, Experimental Cell Research and Academic Press, spring -92.

Supervision of PhD students: Assistant supervisor: Lars Lohikangas, (1995-2000), Main supervisor: Teet Velling (1994-2000, defended thesis 2000-05-18), Carl-Fredrik Tiger (1996-2002, defended thesis 2002-05-16). Current PhD students: Ning Lu registered University of Bergen 2004-05-01, Malgorzata Barczyk to be registered spring 2005

Supervision of postdocs: Wan-Ming Zhang M.D., Ph.D. 00-06-01-02-06-30, Svetlana Popova M.D, Ph.D. 01-09-01 - present, Belen Rodriguez-Sanchez, Ph.D. 03-01-12 - 05-01-30.

Positions and reviewing tasks for granting agencies:

Alliance for Cellular Signaling/Nature (2004 - present) Associate editor on integrins (http://www.signaling-gateway.org/).

Swedish Research council (2003 - present), member of Molecular Cell Biology evaluation committee.

Swedish Research council (2002 - 2004), KOMBI member.
Inflammatory Bowel Disease Program, Eli & Edythe Broad foundation (US) (2002) - Grant review.

Swedish Natural Science Research Council (2000) - External referee for biologikommitten 3.

Wellcome trust (2000, 2003) - Major 5 year grant reviews.

INSERM - (2003-present) External referee to INSERM, France.

Finnish Academy of sciences (1999) - Major Grant review.

Academic expertise: Faculty opponent at thesis defense of Lachmi Jenndahl 05-04-22, Medical Biochemistry, Sahlgrenska Akademien, Gothenburg university

Faculty opponent at thesis defense of Anders Olin 03-06-11 at Dept. of Cell and Molecular biology, Lund University.

Faculty opponent at thesis defense of Michael Stigson 96-11-01 at Inst. f. Miljö och Utvecklingsbiologi, Uppsala University.

"Sakkunning vid docenturansökan", Medical faculty, Lunds university Jan. 2001.

Member of thesis committees since 1995 at least 5/year at Uppsala university (Medical/ Natural Science faculties), Karolinska institute, and Lund University.

Recent conferences: Chairman at session of collagen-binding integrins, Collagen Gordon conference, Boston, June 2003.

Invited speaker to Svenska Föreningen för Biokemi och Molekylärbiologi, Kalmar Sept. 2003.

Chairman at session on developmental biology, Finnish-Swedish Connective tissue society meeting, Åbo, Finland Sept 2003.

Referee for journals: Referee for Cancer Res., Cell & Tissue Res. ,Dev. Dyn., Exp. Cell Res., Eur. J. Cell Biol., Eur. J. Biochem., J. Biol. Chem., J. Cell Sci., Matrix Biol., Mol. Med. 1993 - 2002 invited by journal editor as a regular contributor to Trends in Cell Biology "Headline"section

Patents: Patent application (-99), "use of α11 integrin", patent application initiated by Active Biotech, Lund, currently with Cartela AB, Lund. Patent application (-02), Cartela AB, "use of itga10 and itga11 knockout mice".

Publications last 5 years

1. Popova, S Rodriguez-Sanchez, B.,Lidén. Å., Betsholtz, C., van den Bos, T., Gullberg, D. (2004) The mesenchymal α11β1 integrin attenuates PDGF-BB stimulated chemotaxis of embryonic fibroblasts on collagens. *Dev. Biol.* **270,** 427-442

2. **Gullberg, D.** (2003) Cell biology: the molecules that make muscle *Nature* **424,** 138-140.

3. Zhang, W.-M., Käpyläs, J., Puranen, J.S., Farndale, R., Tiger, C.-F., Pentikäinen, O.T., Johnson, M.S., Heino, J., and **Gullberg, D.** (2003) α11β1 integrin recognizes the GFOGER sequence in interstitial collagens, *J. Biol. Chem.*, **278,** 7270-7278

4. Gullberg D. (2002) Yet another liaison between two cell adhesion families *Trends Biochem. Sci* 27, 602.

5. **Gullberg, D.** (2002) Importance of ECM remodeling clarified *Trends Cell Biol.* **12,** 110.

6. Zhang, W.-M., Bergman,C. , Velling, T., Kusche-Gullberg, M., and **Gullberg, D.** (2002) Analysis of the human integrin α11 gene (ITGA11) and its promoter. *Matrix. Biol.* **21,** 513-523

7. **Gullberg, D.** E., and Lundgren-Akerlund, E. (2002) Collagen-binding I domain integrins--what do they do? *Prog Histochem Cytochem* **37,** 3-54

8. Gullberg, D. (2001) Unconventional gene therapy holds promise for muscle disease *Trends Cell Biol.* **11,** 461.

9. **Gullberg, D.** (2001) Designer integrins proving their value *Trends Cell Biol.* **11,** 193.

10. Gu, Y. C., Talts, J. F., **Gullberg, D.,** Timpl, R., and Ekblom, M. (2001) Glucocorticoids down-regulate the extracellular matrix proteins fibronectin, fibulin-1 and fibulin-2 in bone marrow stroma *Eur J Haematol* 67, 176-184.

11. Tiger, C. F., Fougerousse, F., Grundstrom, G., Velling, T., and **Gullberg, D.** (2001) α11β1 integrin is a receptor for interstitial collagens involved in cell migration and collagen reorganization on mesenchymal nonmuscle cells *Dev Biol* **237,** 116-129.

12. Lohikangas, L., **Gullberg, D.,** and Johansson, S. (2001) Assembly of laminin polymers is dependent on β1-integrins *Exp Cell Res* **265,** 135-144.

13. Champliaud, M. F., Virtanen, I., Tiger, C. F., Korhonen, M., Burgeson, R., and **Gullberg, D.** (2000) Posttranslational modifications and β/gamma chain associations of human laminin α1 and laminin α5 chains: purification of laminin- 3 from placenta *Exp Cell Res* **259,** 326-335.

14. Virtanen, I., **Gullberg, D.,** Rissanen, J., Kivilaakso, E., Kiviluoto, T., Laitinen, L. A., Lehto, V. P., and Ekblom, P. (2000) Laminin α1-chain shows a restricted distribution in epithelial basement membranes of fetal and adult human tissues *Exp Cell Res* **257,** 298-309.

15. Pedrosa-Domellof, F., Tiger, C. F., Virtanen, I., Thornell, L. E., and **Gullberg, D.** (2000) Laminin chains in developing and adult human myotendinous junctions *J Histochem Cytochem* **48,** 201-210.

16. **Gullberg, D.,** Tiger, C. F., and Velling, T. (1999) Laminins during muscle development and in muscular dystrophies *Cell Mol Life Sci* **56,** 442-460.

17. Velling, T., Kusche-Gullberg, M., Sejersen, T., and **Gullberg, D.** (1999) cDNA cloning and chromosomal localization of human α11integrin. A collagen-binding, I domain-containing, β1-associated integrin α-chain present in muscle tissues *J Biol Chem* **274,** 25735-25742.

18. Velling, T., Tiger, C. F., Ekblom, P., and **Gullberg, D.** (1999) Laminin α chains in colon carcinoma cell lines: detection of a truncated laminin α1 mRNA in Caco-2 cells *Exp Cell Res* **248,** 627-633.

BOOK CHAPTERS AND BOOKS

19. **Gullberg, D,** Popova, S.N., and Tiger C-F (2003) Structure and function of α11β1 integrin. In "I domains in integrins" (Ed. **Gullberg, D**), Medical Intelligence Unit of Bioscience Publishers, LANDES Bioscience, Austin, Texas, pp 67-81

MANUSCRIPTS

20. Lu N., Heuchel, R., Barczyk, M., Zhang, W.-M., and **Gullberg, D.** (2005) Tandem Sp1/Sp3 sites together with an Ets-1 site cooperate to mediate α11 integrin chain expression in mesenchymal cells., **submitted**

21. Popova, S., Tiger, C.-F., Barzcyk, M, Beertsen, W., Rozell, B., Rodriguez-Sanchez, R., Forsberg, E., and **Gullberg, D.** (2005) A dwarfed phenotype with tooth defects in mice deficient in integrin α11β1. **manuscript**

22. Grundström, G., Lidén, Å., **Gullberg, D.** and Rubin, K. (2005) Clustering of β1-integrins induces an ERK1/2-dependent αvβ3-mediated collagen gel contraction. **manuscript**

23. Schneller, M., Olsson, A., Åström, U., Pirro-Lundquist, A., Hickery, M., Farndale, R., **Gullberg, D.** and Lundgren-Åkerlund, E. (2005) Opposing regulation of α10β1 and α11β1 on chondrocytes. **manuscript**

24. Tu, H., Rodriguez-Sanchez, B., Popova, S., Zhang, W-M, **Gullberg, D.**, Pihljaniemi, T. (2005) Type XIII collagen is a high-affinity ligand for α11β1 integrin. **manuscript**

25. Mirtti, T., Nylund, C., Lehtonen, J., Hiekkanen, H., Kallajoki, M., **Gullberg, D.** and Heino, J. Collagen receptor integrins in prostate cancer (2005). **submitted to Am. J. Pathol.**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of) Examiner: Maher Haddad

Donald Gullberg) Art Unit: 1644

Serial No. 09/980,403)

Filed: April 15, 2002)

For: "Integrin Heterodimer)
And An Alpha Subunit)
Thereof")

DECLARATION OF DR TEET VELLING

I, Dr Teet Velling, hereby make the following declaration:

1. I am a research scientist at the Department of Medical Sciences, Uppsala University, Uppsala, Sweden

2. My *curriculum vitae* is provided as Enclosure A.

3. I have read and am familiar with US Patent Application No. 09/980,403, which concerns the human integrin alpha11 subunit and its use. I have also reviewed the examination report dated 19 May 2005 issued in connection with US 09/980,403, including the references cited by the examiner.

4. My comments on the merits of the objections raised by the examiner are as follows.

5. The examiner has raised an objection based on the paper by Gullberg et al. 1995, Dev. Dynamics 204, 57-65. I am familiar with this paper as I was involved in the work described in it (and I am named as an author). The paper describes the upregulation of the integrin alphamt subunit on human fetal myotubes.

6. The examiner alleges that the alphamt subunit is identical to the alpha11 subunit described in US 09/980,403. In support of this allegation, the examiner also cites a later paper which I authored, Velling et al., 1999, J. Biol. Chem. 274, 25735-25742.

7. The experiments in the Velling et al paper correspond to the experiments described in the Examples section of US 09/980,403. The inventor, Donald Gullberg, is also an author on this paper.

8. In alleging that the alphamt subunit is "identical" to the alpha11 subunit, the examiner has failed to define what he means by the term "identical". It is my understanding that under patent law, the issue of novelty requires 100% *sequence* identity between the two alpha subunits.

9. The basis of this comparison is highly significant because the alphamt subunit has never been sequenced. It is therefore impossible to conclude that the alphamt subunit shares 100% sequence identity with the alpha11 subunit; this comparison has never been done.

10. One could speculate that the amino acid sequences of the alphamt subunit will be similar to the alpha11 subunit, based on the cloning strategy employed. However, it is simply not possible to say that the amino acid sequence are 100% identical.

11. In my opinion, it is not even possible to say that 100% sequence identity between the alphamt subunit and the alpha11 subunit is *probable* since the two subunits were derived from different tissues. The alphamt subunit is derived from muscle cell precursors (myotubes) while the alpha11 subunit is cloned from a uterus cDNA library. It is quite possible that there will be a degree of sequence variation in such proteins expressed by different tissues.

12. As a consequence, I believe that the examiner's objection based on Gullberg et al. is lacking in scientific basis.

13. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the above-referenced application or any patent issued thereon.

T Velling

Dr Teet Velling

15/11-05

Date

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Education:

- PhD in Animal Physiology, May 2000 with a thesis "Muscle Cell Integrins in vitro and in vivo: Identification and Characterisation of a Novel Integrin, $\alpha 11\beta 1$" under supervision of Dr. Donald Gullberg
- Post Doc in a research group of prof. Staffan Johansson, Dept. Of Medical Biochemistry and Microbiology, Biomedical Centre, Uppsala University (2000-2004).

Present position: research scientist in a group of prof. Agneta Siegbahn, Dept. Of Medical Sciences, Clinical Chemistry, Uppsala University, with 100% time for research. Currently a co-supervisor of two graduate students.

Paternity leave:
September 1, 1998 until December 31, 1998; a forthcoming paternity leave during autumn 2005.

Achievements:
Post Doc:

- characterisation of activation mechanisms of PKB/Akt by epidermal growth factor and β1 integrins (ref 12);
- characterisation of FAK-independent activation of PI3K by integrins (ref. 11)
- characterisation of the role of integrin transmembrane domain in regulation of PI3K-dependent CAS phosphorylation (ref. 10);

- characterisation of a role of integrin-type collagen receptors and fibronectin in collagen polymerisation (ref. 8);

doctoral studies:

- identification and characterisation of the last member of the integrin family – the collagen receptor subunit, integrin α11 (ref-s. 6 and 7);
- detection of a truncated laminin α1 chain in colon carcinoma cell line and partial mapping of the truncation site (ref. 5);
- characterisation of embryonic expression of integrin α7β1 in mouse (ref. 3);

masters studies:

- characterisation of three different monoclonal antibodies produced by immunising mice with whole myogenic cells of human origin;

baccalaureate studies:

- electron microscopic and immunochemical studies on a nematode *Caenorhabditis elegans*.

Participation in scientific conferences:

- October 2004: invited speaker to seminar series at the Department Of Natural Sciences, Tallinn Technical University;
- October 2003: Molecular Biology Of Cellular Interactions, EURESCO conference, Obernai, France. Attendee;
- October 2002: Wenner-Gren Foundation's International Symposium on Cell Signalling – Experimental and Computational Approaches, Stockholm, Sweden. Attendee;
- September 2002: The 5 th SACR meeting on The Cytoskeleton: Dynamics and Signalling Modules, Gimo, Sweden. Speaker;
- July 2002: Gordon Research Conference on Signalling by Adhesion Receptors, New London, USA. Poster presenter;
- February 2002: The International Winter School of Estonian National Institute of Chemical Physics and Biophysics, Kääriku, Estonia. Speaker;
- September 2001: Meeting of the Finnish-Swedish Connective Tissue Society, Stockholm, Sweden. Attendee;
- July 2000: The XVII Meeting of the European Connective Tissue Societies, Patras, Greece. Short talk presenter;
- October 1999: European Research Conference on Molecular Biology of Cellular Interactions: Adhesion Networks and Cytoskeletal Organisation. Castelveccio Pascoli, Italy. Poster presenter;
- March 1999: The First Meeting of the Finnish-Swedish Connective Tissue Society, Turku, Finland. Speaker;
- August 1998: The XVI Meeting of the European Connective Tissue Societies, Uppsala, Sweden. Poster presenter;
- August 1996: The XV Meeting of the European Connective Tissue Societies, Munich, Germany. Poster presenter.

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CMLS Cellular and Molecular Life Sciences

Ligand recognition by the I domain-containing integrins

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Abstract. Seven of the integrin α subunits described to date, α_1, α_2, α_L, α_X, α_d, α_M and α_E, contain a highly conserved I (or A) domain of approximately 200 amino acid residues inserted near the amino-terminus of the subunit. As the result of a variety of independent experimental approaches, a large body of data has recently accumulated that indicates that the I domains are independent, autonomously folding domains capable of directly binding ligands that play a necessary and important role in ligand binding by the intact integrins. Recent crystallographic studies have elucidated the structures of recombinant α_M and α_L I domains and also delineated a novel divalent cation-binding motif within the I domains (metal ion-dependent adhesion site, MIDAS) that appears to mediate the divalent cation binding of the I domains and the I domain-containing integrins to their ligands.

Key words. Adhesion; integrin; I domain; divalent cation; ligand recognition.

Introduction

Of the 15 integrin α subunits that have been identified to date, the α_1, α_2, α_L, α_X, α_d, α_M and α_E subunits contain near their amino-termini an autonomously folded domain of approximately 200 amino acids referred to as the I (inserted) domain. A homologous domain, the A domain, is present in von Willebrand factor, cartilage matrix protein, type IV, VII, XII and XIV collagens, and complement proteins factor B, C2, CR3 and CR4. Von Willebrand factor contains three repeats of the A domain. The first and third repeats have been implicated in collagen binding. This observation led to speculation that the ligand-binding activity of the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins, both of which bind collagens, might also be mediated by their α subunit I domains. Since that time, a substantial body of evidence has accumulated to support the notion that the binding of most, if not all, ligands to I domain-containing integrins is mediated by the integrin α subunit I domain. I domain-mediated ligand binding has been established for five of the seven I domain-containing a subunits. Table 1 summarizes the I domain-containing integrin α subunits, the β subunits with which they associate, their ligands and indicates whether the I domain has been implicated in the ligand-binding activity of the integrin.

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The I domain-containing integrins

$\alpha_1\beta_1$ Integrin

The initial identification and characterization of the $\alpha_1\beta_1$ integrin as a collagen and laminin receptor was an outgrowth of studies with PC12 cells. Pheochromocytoma-derived PC12 cells adhere to laminin and collagen types I, II, III and IV in a Mg^{2+}-dependent manner [1]. The ability of a monoclonal antibody, 3A3, to block the adhesion of PC12 cells to collagens and laminin and to promote the retraction of neurites extended on these substrates led to the identification of the $\alpha_1\beta_1$ integrin as a collagen/laminin receptor [2]. Evidence for the involvement of the α_1 integrin subunit I domain in

Table 1. The I domain-containing integrins.

α Subunit	Parent integrin	Substrate	I domain involvement in ligand binding	References
α_1	$\alpha_1\beta_1$	collagen	yes	[3, 4]
		laminin	yes	[3, 4]
α_2	$\alpha_2\beta_1$	collagen		[20–22, 4]
		laminin	yes	[22, 4]
		collagen C – propeptide	yes	[23]
		echovirus-1	yes	[19,16]
α_L	$\alpha_L\beta_2$	ICAM-1	yes	[38]
		ICAM-2	?	[34]
		ICAM-3	yes	[38]
α_X	$\alpha_X\beta_2$	fibrinogen	?	[55]
		iC3b	yes	[54, 56]
α_d	$\alpha_d\beta_2$	ICAM-3	?	[57]
α_M	$\alpha_M\beta_2$	ICAM-1	yes	[51]
		fibrinogen	yes	[51
		iC3b	yes	[51]
		Factor X	?	[46]
		NIF	yes	[50, 52]
		heparin	yes	[49]
α_E	$\alpha_E\beta_4$	?	?	[59, 60]
	$\alpha_E\beta_7$	?	?	[62–64]

ligand binding was obtained using another $\alpha_1\beta_1$ integrin function-blocking antibody, 1B3.1. This antibody failed to bind to a functional chimeric $\alpha_1\beta_1$ integrin that consisted of the chicken I domain inserted into the human α_1 subunit. Furthermore, mutation of D253 within the chicken I domain to alanine restored 1B3.1 binding and caused a significant decrease in cell adhesion to collagen IV and laminin [3]. Finally, recombinant α_1 integrin I domain was shown to bind to types I and IV collagen and laminin in a divalent cation-dependent manner that was inhibited by an $\alpha_1\beta_1$ integrin function-blocking antibody [4]. Thus the adhesion of cells to collagens and laminin via the $\alpha_1\beta_1$ integrin appears to be dependent upon interactions involving the α_1 I domain.

$\alpha_2\beta_1$ Integrin

Recognition that the $\alpha_2\beta_1$ integrin serves as a collagen/laminin receptor grew out of early studies with platelets [5]. Platelets adhere to collagen in a divalent cation-dependent manner [6]. Mg^{2+}, Mn^{2+} and several other divalent cations support the adhesion of platelets to collagen. Ca^{2+}, which fails to support platelet adhesion to collagen, inhibits the Mg^{2+}-dependent adhesion. Following surface iodination and membrane protein solubilization, a 160,000-Da protein was identified as the mediator of cation-dependent adhesion of platelets to collagen [6]. A heterodimeric complex containing the 160,000-Da protein and an additional 130,000-Da protein was purified from platelet membranes by sequential chromatographic steps on concanavalin A-Sepharose and collagen-Sepharose. This complex was shown by immunological means to be identical to VLA-2, or what is now known as the $\alpha_2\beta_1$ integrin [7]. Further evidence that the $\alpha_2\beta_1$ integrin was a Mg^{2+}-dependent collagen-binding protein was obtained when the purified integrin was incorporated into liposomes. Like cells expressing the $\alpha_2\beta_1$ integrin, the liposomes bound collagen in an Mg^{2+}-dependent manner that was inhibited by Ca^{2+} [7, 8]. Other independent investigations carried out originally with platelets and HT-1080 cells identified the $\alpha_2\beta_1$ integrin as the antigen recognized by an antibody that inhibited cell adhesion to collagen [9–11]. Platelet deficiency of the $\alpha_2\beta_1$ integrin results in platelet unresponsiveness to collagen and a bleeding disorder [12].

Although the $\alpha_2\beta_1$ integrin is widely expressed on several different cell types, its substrate specificity varies. While it is a receptor for both collagen and laminin on endothelial and epithelial cells [13, 14], it binds only collagen when expressed on platelets [8]. The identification of a monoclonal antibody directed against the β_1 integrin that is capable of converting the $\alpha_2\beta_1$ integrin from a collagen-binding form to a collagen/laminin-binding form provides compelling evidence that the difference between these two forms of the integrin is their conformational state [15]. In addition to the extracellular matrix components collagen and laminin, the $\alpha_2\beta_1$ integrin has been shown to be a receptor for a common human viral pathogen, echovirus-1 [16], and the carboxyl-terminal propeptide of type I collagen [17].

Considerable evidence exists to implicate the I domain of the α_2 integrin subunit in the binding of the $\alpha_2\beta_1$ integrin with its ligands. An antiserum directed against an α_2 I domain fusion protein expressed in bacteria disrupts the adhesion of endothelial cells to type I collagen and laminin [18]. Using a series of human/bovine α_2-integrin subunit chimeras, the epitopes of all known $\alpha_2\beta_1$ function-blocking monoclonal antibodies have been mapped to the I domain [19]. Point mutations at several critical residues within this region of the α_2 subunit abolish the adhesion of cells and the binding of recombinant I domain to collagen [19, 20]. Recombinant α_2-integrin I domain specifically binds collagen, laminin and the carboxyl-terminal propeptide of type I procollagen in a divalent cation-dependent manner [4, 21–23]. Thus, as is the case for the $\alpha_1\beta_1$ integrin, the I domain of the α_2 integrin subunit is the binding site of all known ligands of the $\alpha_2\beta_1$ integrin.

β_2 Integrin subfamily

The β_2 integrins consist of four adhesion receptors whose expression is restricted to white blood cells. Unlike the β_1 integrins, all known β_2 integrins contain an I domain within their α subunits. Within this group are the $\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$ and $\alpha_d\beta_2$ integrins. An inherited β_2-integrin deficiency has provided evidence for the importance of the β_2 integrins for normal immune function. Leukocyte adhesion deficiency (LAD) is a disease in which the expression of the β_2-integrin subunit is either deficient or absent [24]. The loss (or decreased expression) of the β_2 integrins in LAD patients results in recurring bacterial infections, impaired neutrophil mobility and respiratory burst, abnormal neutrophil phagocytosis and other related defects. Transfection of the β_2-integrin subunit complimentary DNA (cDNA) into B lymphoblastoid cells from LAD patients restored normal levels of the $\alpha_L\beta_2$ integrin to the cell surface [25]. Homotypic adhesion and adhesion to intercellular adhesion molecule-1 (ICAM-1) was also restored in the β_2 transfected cells. These experiments verified that the LAD defect is in the β_2-integrin subunit.

$\alpha_L\beta_2$ Integrin

The $\alpha_L\beta_2$ integrin, also referred to as LFA-1 (lymphocyte function-associated antigen 1), is expressed on all leukocytes with the exception of some macrophages [26]. Although identified by screening for monoclonal antibodies that inhibited CTL (cytolytic T lymphocyte)-mediated killing [27], the $\alpha_L\beta_2$ integrin is also required for many other leukocyte functions, including T-helper and B-lymphocyte responses, natural killing, antibody-dependent cytotoxicity mediated by monocytes and granulocytes, and adhesion of leukocytes to endothelial cells, fibroblasts and epithelial cells [28].

The first counterreceptor identified for the $\alpha_L\beta_2$ integrin, ICAM-1, was identified by screening for monoclonal antibodies that inhibited the aggregation of $\alpha_L\beta_2$-positive cells [29]. ICAM-1 expression is rapidly induced by inflammatory cytokines [30], and analysis of its cDNA sequence has revealed that it is a member of the immunoglobulin supergene family with five immunoglobulin-like domains [31, 32]. The identification of ICAM-1 as a ligand for the $\alpha_L\beta_2$ integrin provided the first example of an interaction between a member of the integrin family and a member of the immunoglobulin superfamily.

The adhesion of inflammatory cells to endothelial cell monolayers occurs by both $\alpha_L\beta_2$-dependent and $\alpha_L\beta_2$-independent mechanisms. $\alpha_L\beta_2$-Dependent adhesion is further subdivided into $\alpha_L\beta_2$-dependent/ICAM-1-dependent and $\alpha_L\beta_2$-dependent/ICAM-1-independent mechanisms [33]. This led to the search for additional ligands for the $\alpha_L\beta_2$ integrin. ICAM-2, identified by functional screening of a cDNA library prepared from endothelial cells [34], is also a member of the immunoglobulin superfamily. ICAM-2 contains two immunoglobulin-like domains. These domains are similar (34% identity) to immunoglobulin-like domains 1 and 2 of ICAM-1. Unlike ICAM-1, the basal expression of ICAM-2 on endothelial cells is high, and its expression is not induced further by lipopolysaccharide [34].

The observation that a mixture of blocking monoclonal antibodies to ICAM-1 and ICAM-2 failed to inhibit completely the adhesion of some T- and B-lymphocyte cell lines to purified $\alpha_L\beta_2$ integrin was the impetus for a search for a monoclonal antibody that could inhibit the remaining adhesion, thus identifying a third ligand for the $\alpha_L\beta_2$ integrin [35]. Such an antibody was identified; adhesion of resting lymphocytes to purified $\alpha_L\beta_2$ integrin was almost entirely mediated by ICAM-3. Cloning of the ICAM-3 cDNA revealed that it is also closely related to ICAM-1 [36, 37]. Like ICAM-1, ICAM-3 contains five immunoglobulin-like domains. Unlike ICAM-1, however, ICAM-3 is constitutively expressed on resting leukocytes, leading to the speculation that it may be important for the initiation of the immune response [36].

Evidence for the involvement of the α_L I domain in binding to ICAM-1 and ICAM-3 has been obtained. A screen of 21 α_L and 10 β_2 monoclonal antibodies identified a single α_L antibody, MEM-83, that was capable of enhancing the binding of T cells to purified ICAM-1. Using a series of α_L-deletion mutants, the MEM-83 activation epitope was mapped to the I domain [38]. In addition, purified recombinant α_L-integrin I domain binds to ICAM-1 and inhibits $\alpha_L\beta_2$-integrin-dependent T cell adhesion to ICAM-1. The epitopes of 18 out of

20 $\alpha_L\beta_2$ integrin-function blocking antibodies map to the I domain [39]. Finally, a screen of 19 α_L and 10 β_2 monoclonal antibodies identified two α_L antibodies that inhibited T-cell adhesion to ICAM-3 [40]. The epitopes of both of these antibodies were mapped to the I domain. Interestingly, neither of these antibodies inhibited T-cell adhesion to ICAM-1. Conversely, the activating antibody MEM-83 was incapable of stimulating T-cell adhesion to ICAM-3. The authors speculate that alternative forms of the I domain may be present on the cell surface after activation and that the activating antibody is capable of inducing or stabilizing the ICAM-1-binding form, while the inhibitory antibodies interfere with the ICAM-3-binding form. In any event, these studies provide data that demonstrate the importance of the α_L I domain with respect to ligand binding of the $\alpha_L\beta_2$ integrin.

$\alpha_M\beta_2$ Integrin

The adhesive function of the $\alpha_M\beta_2$ integrin, or Mac-1, was identified using a monoclonal antibody (M1/70) from a panel of antibodies that reacted with mouse cell surface antigens [41]. The distribution of the $\alpha_M\beta_2$ integrin is more restricted than that of the $\alpha_L\beta_2$ integrin; it is found almost exclusively on myeloid cells [42]. The ability of antibody M1/70 to inhibit rosetting of iC3b-coated erythrocytes to murine macrophages and human polymorphonuclear leukocytes identified the $\alpha_M\beta_2$ integrin as the type three complement receptor [43]. Activated neutrophils adhere to stimulated endothelial cells in a manner dependent on the $\alpha_M\beta_2$ integrin on the neutrophil and ICAM-1 on the endothelial cells, demonstrating that ICAM-1, a ligand for the $\alpha_L\beta_2$ integrin, also binds the $\alpha_M\beta_2$ integrin [44]. A role of the $\alpha_M\beta_2$ integrin/ICAM-1 interaction is to facilitate firm attachment of leukocytes to the endothelium following rolling along the endothelium at sites of inflammation [45]. Stationary adhesion is a prerequisite for neutrophil extravasation at sites of inflammation. Additional ligands for the $\alpha_M\beta_2$ integrin include factor X [46], fibrinogen [47, 48] and heparin [49]. The hookworm-derived glycoprotein NIF (neutrophil inhibitory factor) exerts its effects of blocking both the adhesion of activated neutrophils to vascular endothelial cells and the release of H_2O_2 from activated neutrophils by virtue of a high-affinity interaction with the $\alpha_M\beta_2$ integrin [50].

Most of the $\alpha_M\beta_2$ integrin/ligand interactions that have been characterized to date are clearly due to recognition of the ligands by the α_M-integrin subunit I domain. The epitopes recognized by monoclonal antibodies that block the binding to $\alpha_M\beta_2$ of iC3b, ICAM-1, fibrinogen and a fourth ligand that facilitates neutrophil homotypic adhesion all map to the α_M integrin I domain [51]. NIF binds recombinant α_M I domain with high affinity and in a specific manner. The binding is blocked by a monoclonal antibody directed against the α_M I domain [52].

$\alpha_X\beta_2$ Integrin

Expression of the $\alpha_X\beta_2$ integrin, previously referred to as p150,95, is mainly restricted to myeloid cells [53]. The isolation of activated U937 (a monocytic cell line) cell surface proteins by affinity chromatography on immobilized iC3b led to the identification of the $\alpha_X\beta_2$ integrin as an iC3b receptor [54]. Tumour necrosis factor-α-stimulated polymorphonuclear leukocytes adhere to fibrinogen in a manner blocked by two monoclonal antibodies directed against the α_X-integrin subunit [55]. Thus fibrinogen is a ligand for both the $\alpha_M\beta_2$ and the $\alpha_X\beta_2$ integrins. The epitope recognized by an α_X-integrin monoclonal antibody capable of blocking the adhesion of cells and the binding of purified $\alpha_X\beta_2$ integrin to iC3b has been mapped to the α_X-integrin subunit I domain, showing the importance of this I domain in the ligand recognition and binding by the $\alpha_X\beta_2$ integrin [56].

$\alpha_d\beta_2$ Integrin

The most recently identified member of the β_2-integrin subfamily is the $\alpha_d\beta_2$ integrin [57]. The α_d subunit is more similar to the α_M and α_X integrin subunits than to the α_L subunit. The distribution of the $\alpha_d\beta_2$ integrin is somewhat different than that of the $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrins. It is expressed on macrophages and granulocytes in the splenic red pulp, on lipid-laden macrophages in aortic fatty streaks and to a lesser extent on peripheral blood leukocytes. Flow cytometry and solid-phase adhesion assays were used to compare the binding of the $\alpha_d\beta_2$ integrin to ICAM-1 and ICAM-3. Both methods demonstrated that the $\alpha_d\beta_2$ integrin preferentially bound ICAM-3. Involvement of the α_d-integrin subunit I domain in ligand recognition and binding has not been demonstrated.

$\alpha_E\beta_4$ and $\alpha_E\beta_7$ integrins

The screening of monoclonal antibodies prepared against the human pancreatic cell line FG led to the discovery of the $\alpha_E\beta_4$-integrin [58, 59]. This integrin is localized to the basal surface of keratinocytes, and anti-β_4-integrin antibodies block the adhesion of keratinocytes to purified laminin [60]. Thus the $\alpha_E\beta_4$ integrin appears to mediate the adhesion of keratinocytes to basal lamina. Specific ligands for the $\alpha_E\beta_4$ integrin have not been identified.

The $\alpha_E\beta_7$ integrin was also identified by reactivity with a monoclonal antibody [61]. This integrin, along with $\alpha_4\beta_7$,

is expressed on intestinal intraepithelial lymphocytes. The $\alpha_E\beta_7$ integrin, in conjunction with the $\alpha_L\beta_2$ integrin, has been shown to mediate the adhesion of T lymphocytes to epithelial cells [62]. The epithelial cell counterreceptor for the $\alpha_E\beta_7$ integrin has been identified as E-cadherin [63]. Cloning of the α_E-integrin subunit revealed the presence of an I domain, and an extra 55 amino acid domain just amino-terminal to the I domain [64]. This extra domain is highly negatively charged and contains a protease cleavage site. Among the α-integrin subunits, this domain is unique to α_E. Northern blot analysis revealed that the α_E-integrin messenger RNA (mRNA) is restricted to mucosal lymphocytes. As yet there is no evidence for the involvement of the α_E I domain in ligand binding.

I domain structure

Protein structure

The primary sequence of each of the integrin α-subunit I domains is shown in figure 1. Even a cursory examination of the sequences reveals that the integrin I domains constitute a family of closely related proteins with a high degree of sequence identity and/or similarity. Recent efforts by two groups have led to solution of crystal structures for the α_M- and α_L-integrin subunit I domains [65–68]. These studies have resulted in dramatic new insights into I domain structure and function.

As the major structural features identified were common to both the α_M and α_L I domains, only the α_M structure is described in detail. A stereoscopic ribbon diagram of the α_M domain is presented in figure 2. The I domain is composed of alternating amphipathic α helices and hydrophobic β strands. The five parallel and one antiparallel β strands form a central sheet that is surrounded by the seven α helices. The I domain structure is an example of the 'Rossmann' dinucleotide, or doubly-wound fold. A crevice is formed along the top of the β sheet. In all other proteins exhibiting this type of folded structure, the apical crevice functions as a ligand-binding site. Additional observations described below suggest that this is probably also true for the I domains.

Divalent cation binding

In accordance with observations from an earlier experimental study suggesting that the I domain contained a novel divalent cation-binding motif [69], a single divalent cation binding site was located in the α_M and α_L I domain crystal structures in the crevice at the top of the β sheet. The structural basis of Mg^{2+} binding to the α_M I domain will be used as an example to facilitate an initial detailed discussion of the metal-binding properties of the I domain (fig. 3a).

Mg^{2+} has sites for six coordinating ligands arranged in an octahedral geometry. Three of the six Mg^{2+} coordination sites in the I domain are provided by the hydroxyl oxygen atoms of S142, S144 and T209 of α_M. Two additional coordination ligands are provided by water molecules w1 and w2. w2 is also hydrogen bonded to a carbonyl oxygen of E244. The two aspartate residues, D140 and D242, previously implicated in metal binding by mutagenesis studies, appear to participate indirectly by hydrogen bonding to a water molecule (w1) and hydroxyl side chains of S142 and S144, respectively. Somewhat surprisingly, the sixth and apical coordination ligand was provided by E314 of a neighbouring I domain molecule in the crystal. It seems likely that, in solution, this coordinating ligand is provided by water. Given the position of the metal ion in the putative ligand-binding crevice, it is attractive to consider that an acidic side chain from the ligand might provide a site in the ligand-bound form of the I domain.

The α_M I domain has also been crystallized in the presence of Mn^{2+} [66]. The profound structural differences observed between the Mn^{2+}- and Mg^{2+}-occupied forms of the I domain may shed light on the structural basis underlying some divalent cation-dependent alterations in I domain ligand-binding specificity. For example, Mg^{2+} effectively supports binding of the α_2-integrin I domain to collagen and only poorly supports binding to laminin, whereas Mn^{2+} effectively supports binding to both ligands [22, 70]. The manner in which Mn^{2+} is coordinated in the α_M I domain is shown in figure 3b. The position of the divalent cation is shifted by 2.3 Å, relative to its position in the Mg^{2+}-loaded form of the I domain. The two serine residues continue to provide coordinating ligands, as in the Mg^{2+} form. However, in the Mn^{2+} form, the coordinating side chain of T209 is replaced by the D242 side chain. The three remaining sites are provided by water molecules. The changes in coordination pattern have marked effects on the protein structure. The C-terminal α7 helix (see fig. 2) moves 10 Å up the side of the I domain molecule, with burial of F302, which was completely exposed in the Mg^{2+} structure. These changes necessitate additional movements of three connecting segments, subsequent burial of F275, which was also exposed in the Mg^{2+} structure, and consequent repositioning of D242 so that it is now capable of supplying a coordination ligand to the Mn^{2+}. Although the α_M I domain structure is profoundly dependent upon the identity of the bound divalent cation, this does not appear to be the case for the α_L I domain. Structures of the α_L I domain have recently been solved for crystals containing either Mg^{2+} or Mn^{2+}, as well as for I domains free of metal [68]. Although the general

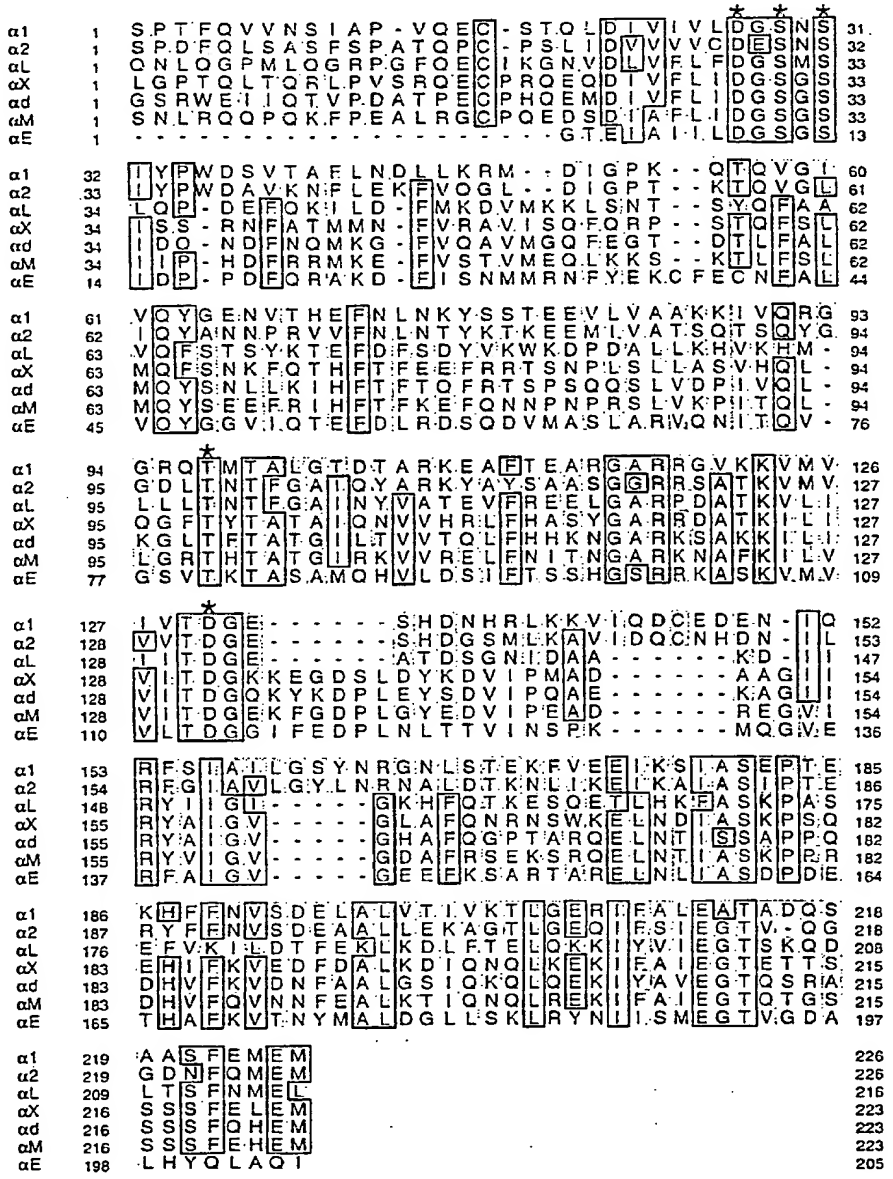

Figure 1. Comparison of amino acid sequences of the I domains from the seven I domain-containing integrins. Regions of identity are boxed; regions of identity or conserved similarity are shaded. Positions of the conserved residues constituting the MIDAS motif are indicated by asterisks.

features of I domain structure from the study of the α_M domain clearly apply to the α_L I domain, the α_L structure was not dependent upon the presence or identity of divalent cation. The α_L data suggest that the divalent cation dependence of ligand binding and specificity arises either because of direct interactions of ligands with the metal, or because the metal is required to promote a favourable quaternary arrangement of the integrin [68].

A recent study of divalent cation binding to the recombinant α_2-integrin subunit I domain exploited Tb^{3+}, a fluorescent probe of protein divalent cation-binding sites, to examine the relative affinities of divalent cations for the I domain (S. K. Dickeson et al., unpub-

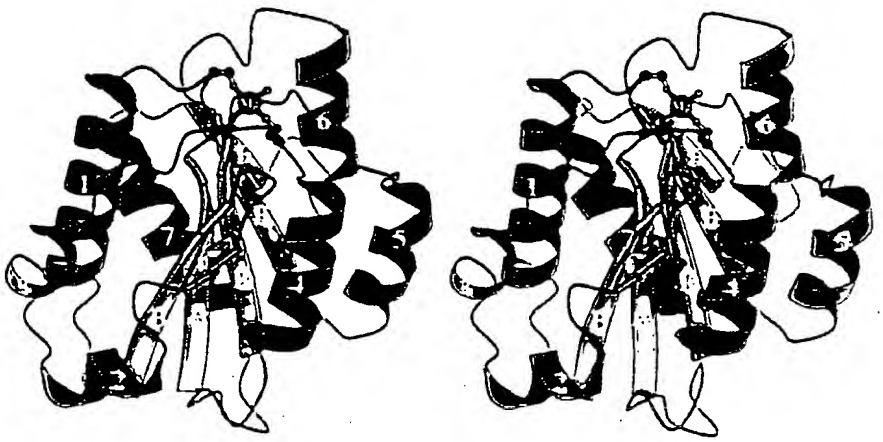

Figure 2. Schematic stereoribbon diagram of the α_M integrin subunit I domain structure. Reprinted with permission from: Lee J.-O., Rieu P., Arnaout M. A. and Liddington R. (1995). Crystal structure of the A domain from the α subunit of integrin CR3 (CD11b/CD18). Cell 80: 631–638, © Cell Press, 1997.

lished observations). As expected from earlier studies, Ca^{2+} did not compete with Tb^{3+} for binding. Although Mg^{2+} and Mn^{2+} both competed with Tb^{3+} for binding, Mn^{2+} was a much more effective competitor than Mg^{2+}, suggesting that the α_2-integrin I domain divalent cation-binding site prefers Mn^{2+} over Mg^{2+}.

The above studies have placed the divalent cation-binding properties of the integrin I domains on a very solid structural foundation. Metal ion binding is mediated by the conserved DXSXS sequence, residues 140–144 in α_M and homologous regions in other I domains, T209 and D242 of α_M and their equivalents in other I domains. Unlike the EF-hand metal-binding motifs present elsewhere in integrin a subunits, the I domain metal-binding motif is constructed from non-contiguous residues. The DXSXS motif and the equivalents of T209 and D242 of α_M are conserved in all integrin I domains. The locations of these conserved metal-binding residues are indicated by asterisks in figure 1. The function of these residues in divalent cation and ligand binding is supported by rather extensive mutagenesis studies of the α_M-, α_L- and α_2-integrin subunits [19, 20, 69, 71]. Although mutagenesis of T221 of the α_2 I domain was initially thought to impair direct interaction with collagen [20], T221 of α_2 is homologous to T209 of α_M. The divalent cation dependence of ligand binding to integrins and their I domains, as well as the location of the motif in a putative ligand-binding crevice of the I domain, has led to its designation as a MIDAS (metal ion-dependent adhesion site) motif [65].

Ligand binding

Rapid and dramatic progress has been made in identifying potent, low molecular weight antagonists of ligand binding for many of the integrins that lack I domains. Similar attempts to identify small inhibitory peptides derived from the ligands of I domain-containing integrins have met with only limited success, resulting in the identification of only weakly inhibitory sequences [72–74]. It now seems likely that most I domain-containing integrins will be found to recognize structurally and conformationally complex determinants within their ligands that are not effectively mimicked by short linear synthetic peptides.

Although abundant evidence has now accumulated to support the roles of the I domain and its MIDAS motif in ligand binding to I domain-containing integrins, a substantial body of data indicates that amino acid residues and structural regions other than those mediating divalent cation binding are essential for ligand binding and that the different ligands for a given I domain may interact with the I domain somewhat differently. Only a few pertinent examples are cited to support these concepts.

The human α_2 integrin and I domain bind collagens, laminin and echovirus 1 [75]. Antibodies that inhibit binding to collagen and laminin have no effect on echovirus binding and vice versa, despite the fact that all of the antibodies map to epitopes within the I domain [19, 76]. Furthermore, although the human and mouse α_2 integrin subunits are 84% identical, the human integrin binds echovirus, whereas the mouse integrin does not. Insertion of the human α_2-integrin I domain into the murine α_2-integrin subunit to create a murine/human

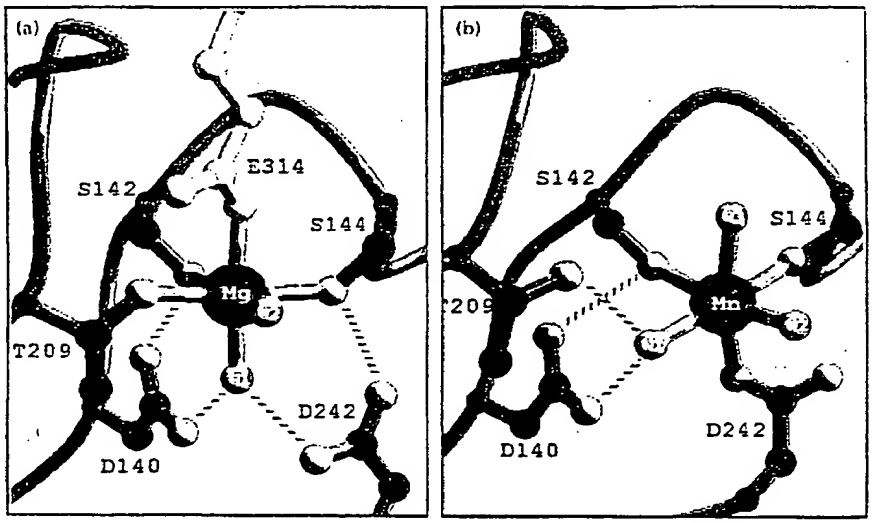

Figure 3. High-resolution comparison of divalent cation coordination and I domain structure of the Mg^{2+} (*a*) and Mn^{2+} (*b*) forms of the α_M-integrin subunit I domain. Water molecules are labelled w1–w3. Selected hydrogen ions are shown by dashed lines. The identity of coordinating amino acid side chains are indicated by a single letter code. Reprinted with permission from: Lee J. -O., Bankston L. A., Arnaout M. A. and Liddington R. C. (1995). Two conformations of the integrin A-domain (I-domain): a pathway for activation? Structure 3: 1333–1340, © Current Biology Ltd., 1997.

chimeric α_2 subunit containing the human I domain conferred virus-binding activity upon the chimera [76]. Although the α_2-integrin I domain binds both collagens and laminin, type I collagen binding was markedly enhanced by the addition of the contiguous first EF-hand motif to the recombinant I domain, whereas laminin binding was unaffected by this addition [22, 70].

As indicated earlier, the I domain of the α_M subunit is a major recognition site for four distinct $\alpha_M\beta_2$ ligands: iC3b, fibrinogen, ICAM-1 and an uncharacterized counterreceptor responsible for neutrophil homotypic adhesion. The use of a battery of monoclonal antibodies that recognized distinct epitopes within the I domain and blocked the binding of one or more ligands to the integrin led to the conclusion that ligand recognition sites in the I domain were overlapping but nonidentical, as individual $\alpha_M\beta_2$-ligand pairs were distinguishable by discrete patterns of antibody inhibition [51]. A recent mutagenesis experiment reinforces this conclusion. A switch of the R281QELNTI sequence in helix 6 of the α_M I domain to the corresponding QETLHKF sequence of the α_L subunit completely inhibited adhesion to fibrinogen, but had no effect on iC3b or NIF binding by the integrin [77]. A very recent study has localized the NIF-binding site to a limited region on the surface of the α_M I domain composed of P147-R152, P201-K217 and D248-R261 [78]. These regions are located in the α1 helix, the connecting segment between the α3 and α4 helices, and helix α5, respectively, in the model shown in figure 2. When these regions of the α_M I domain were introduced into the α_X I domain, the α_X I domain acquired high-affinity NIF-binding activity.

Similar but less extensive studies have been carried out to examine the binding of $\alpha_L\beta_2$ to ICAM-1 and ICAM-3. Two monoclonal antibodies that selectively blocked binding to ICAM-3 but had no effect on binding to ICAM-1 were identified, both of which recognized epitopes within the I domain [40]. A third antibody recognizing a distinct I-domain epitope activated $\alpha_L\beta_2$ to enhance binding to ICAM-1 but not ICAM-3.

Summary and conclusions

A large body of data obtained during the past few years using multiple independent experimental approaches has unambiguously established a crucial role for the I domain in the binding of ligands by the intact I domain-containing integrins. A novel type of divalent cation binding site, the MIDAS motif, has been defined within the I domain and is thought to play a critical role in the divalent cation-dependent binding of I domains and I domain-containing integrins to their ligands. Our recent observations suggest that binding of at least some ligands to the α_2-integrin I domain results in displacement of the divalent cation from the integrin (S. K. Dickeson et al., unpublished observations).

Recent crystallographic studies of the α_{M} and α_{L} I domains have considerably elevated our understanding of I domain structure and have provided clues to design more sophisticated approaches to define the detailed molecular interactions involved in ligand binding to the I domains. The next large step in our understanding of these biologically important receptor-ligand interactions will likely be a three-dimensional view of an I domain-ligand complex.

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EXPERIMENTAL CELL RESEARCH 190, 254-264 (1990)

Different β_1-Integrin Collagen Receptors on Rat Hepatocytes and Cardiac Fibroblasts

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Detergent extracts of primary rat hepatocytes and neonatal cardiac fibroblasts were applied to collagen type I–Sepharose in the presence of 1 m*M* $MnCl_2$. Elution of bound proteins by 10 m*M* EDTA yielded one β_1-integrin heterodimer from hepatocytes with an M_r of 180,000/115,000 under nonreducing conditions. Two β_1-integrins with M_r's (nonreduced) of 180,000/115,000 and 145,000/115,000 could be isolated from surface-iodinated fibroblasts. A monoclonal antibody, 3A3, directed against the rat homolog of the human integrin VLA-1, precipitated the affinity-purified M_r 180,000/115,000 heterodimer, establishing the relatedness of the M_r 180,000 subunit to the α_1-chain of the β_1-integrin subfamily. Both the $\alpha_1\beta_1$-integrin and the 145,000/β_1-integrin heterodimers bound specifically to Sepharose beads derivatized with the collagen fragment α1(I) CB3, which lacks RGD sequences. Immunofluorescence staining using the 3A3 monoclonal antibody revealed that the rat $\alpha_1\beta_1$-integrin was present at focal adhesion sites of fibroblasts grown on native collagen type I- but not on fibronectin-coated substrates, although both types of substrates supported the formation of β_1-integrin containing focal adhesions. Similarly, hepatocytes cultured on substrata coated with collagen type I (but not fibronectin) were stained in a patchy pattern localized to the cell periphery by 3A3 IgG. Furthermore, 3A3 IgG completely inhibited the attachment of hepatocytes to collagen type I, whereas under identical conditions the attachment of fibroblasts to these substrates was inhibited only by approximately 40%. The attachment of both hepatocytes and cardiac fibroblasts to fibronectin was unaffected by the presence of the 3A3 antibody. Collectively these data show that a rat homolog of the human VLA-1 heterodimer both biochemically and functionally fulfills the criteria of a single collagen receptor on rat hepatocytes. In contrast, rat cardiac fibroblasts utilize two different collagen-binding integrins to adhere to collagen, one of which is the rat homolog of the human VLA-1 heterodimer. Furthermore α1(I) CB3 contains cell binding sites for β_1-integrins. © 1990 Academic Press, Inc.

INTRODUCTION

Integrins are a family of cell surface receptors which structurally are heterodimers composed of noncovalently linked α- and β-chains [1, 2]. On the basis of different β-chain compositions the integrin family can be divided into at least four subfamilies, named β_1–β_4 integrins [1–4]. Within each subfamily the same β-chain is associated with a number of distinct α-chains, thought to be determinants of ligand selectivity. The nomenclature of the VLA-1 through VLA-6 proteins, with corresponding α-chains, α_1 through α_6 [4, 5], is commonly used to designate members of the β_1-integrin subfamily, which includes fibronectin receptors [6–9], laminin receptors [10–13], and collagen receptors [8, 9, 14, 15]. Integrins can be classified as Arg–Gly–Asp (RGD)-dependent or RGD-independent, respectively. The RGD-dependent integrin matrix receptors include receptors for fibronectin, vitronectin, von Willebrand factor, fibrinogen, thrombospondin, bone sialoprotein, and tenascin [1, 2, 16–18].

Recent data have suggested that the integrin system can utilize a number of mechanisms to modulate cellular adhesiveness to the extracellular matrix. Thus, multiple integrin receptors exist for individual matrix ligands [6–15]. The expression of integrins is altered by cytokines [19, 20] and cellular transformation [21]. Alternative splicing has been reported for *Drosophila* integrins [22] and mammalian integrins [23], and the existence of cell-specific modulatory factors that can determine the ligand selectivity of integrin heterodimers has recently been postulated [13]. Further complexity is introduced into this system by the finding that α-chains within one subfamily can associate with β-chains of another subfamily [4, 24–26]. To understand the complexity of cell adhesion to individual matrix components and the cellular regulation of these events, it is important to

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establish the basic characteristics of particular receptor ligand interactions. The present report is a continuation of our earlier work aiming at characterizing the interaction of primary hepatocytes and cardiac fibroblasts with collagen at the molecular level [15, 27–29].

The RGD tripeptide is present numerous times in several of the genetically different collagen types [30–35]. However, many cell types seem to bind native collagen in an RGD-independent manner [8, 14, 15, 36, 37] and RGD-containing peptides fail to elute integrin receptors from collagen–Sepharose [14, 38]. The attachment of many cell lines to denatured collagen type VI is, however, inhibited by RGD-containing synthetic peptides [34]. In the present report we show that the α1(I)CB3 fragment, which lacks RGD, contains binding sites for β_1-integrins. We also demonstrate, both biochemically and functionally, that primary rat hepatocytes possess a single collagen-binding integrin receptor. This collagen-binding integrin heterodimer has an M_r of 180,000/115,000 and is the rat homolog of the human VLA-1 integrin heterodimer. Primary rat cardiac fibroblasts, by contrast, possess two collagen-binding integrins, one of which is the rat homolog of the human VLA-1 heterodimer.

MATERIALS AND METHODS

Reagents. Calf collagen type I was obtained commercially (Vitrogen 100, Collagen Corp., Palo Alto, CA). GRGDS peptides were from Bachem Feinchemikalien AG (Switzerland). GRGDTP and GRGESP peptides were from Telios Pharmaceuticals (San Diego, CA). CNBr-activated Sepharose CL-4B, Protein A–Sepharose CL-4B, and S Sepharose Fast Flow were from Pharmacia AB (Uppsala, Sweden). Nitrocellulose paper was from Schleicher & Schüell. Lactoperoxidase, goat anti-mouse IgG coupled to Sepharose, glucose oxidase type II, trypsin type III, α-chymotrypsin type II, and pepstatin A were from Sigma Chemical Co. (St. Louis, MO). Leupeptin and staphylococcal V8 endoproteinase were from Boehringer-Mannheim (Mannheim, FRG) and aprotinin was from Bayer (Leverkusen, FRG). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Fab was purchased from ICN Immuno Biological (Lisle, IL). Texas Red avidin D, biotinylated horse anti-mouse IgG, and biotinylated goat anti-rabbit IgG were from Vector (Burlingame, CA), rabbit anti-mouse IgG was from Zymed (San Francisco, CA), and FITC-conjugated goat anti-rabbit IgG was from Sigma.

Preparation of CNBr peptides of calf collagen type I. Calf collagen type I (100 mg) was dissolved in 20 ml of 70% (v/v) formic acid containing CNBr (12 mg/ml) and digested at room temperature overnight. Digestion was terminated by dilution with water and freeze drying. The cleaved product was then subjected to cation-exchange chromatography on 20 ml of S Sepharose Fast Flow at room temperature as described [39]. Fractions from the chromatography were subjected to 10–15% SDS–PAGE and stained with Coomassie blue. Fractions eluting at the position expected of α1(I) CB3 and with the apparent molecular weight in SDS–PAGE, corresponding to a pure sample of α1(I) CB3 kindly provided by Dr. Rupert Timpl (Munich, FRG), were pooled and freeze-dried. The pooled CB3 was free of contaminants as judged by silver staining.

Preparation of affinity columns. Rat skin collagen type I, or alternatively calf collagen type I or calf α1(I) CB3, was coupled to CNBr-activated Sepharose CL-4B at a concentration of 2 mg/ml in 0.26 *M* NaCl, 20 m*M* NaH_2PO_4, pH 8.2, at 4°C. Sepharose substituted with the M_r 105,000 cell binding fragment of fibronectin was donated by Dr. Staffan Johansson [40].

Cells. Hepatocytes were obtained by a collagenase-perfusion method as described by Öbrink [41]. Monolayer cultures of primary neonatal rat cardiac fibroblasts were established by differential attachment of cells obtained after collagenase digestion of hearts as previously described [42]. Cardiac fibroblasts were grown in DMEM medium supplemented with 10% fetal bovine serum and used between passages 2 and 10.

Antibodies. Anti-β_1-integrin IgG was made as described [15]. Polyclonal antibodies to the M_r 180,000 hepatocyte protein were made as described below. Briefly, collagen-binding integrins from rat hepatocytes were purified as described under *Affinity chromatography*. EDTA-eluted material was pooled, dialyzed against water in the cold, lyophilized, subjected to 99% EtOH at −20°C to remove detergent, and separated on a 5–10% SDS–polyacrylamide gel under nonreducing conditions. After completed electrophoresis the gel was stained briefly with Coomassie brilliant blue, and the gel segment containing the M_r 180,000 protein was cut from the gel and subjected to electroelution as described [15, 43]. Electroeluted protein was dialyzed against 0.13 *M* NaCl, 0.01 *M* Tris–HCl, pH 7.4 (TBS), and used to immunize a rabbit subcutaneously and intradermally in the thighs and on the back until a stable titer was obtained as judged by immunoprecipitation of surface-iodinated fibroblasts. IgG was purified from immune sera by affinity chromatography on Protein A–Sepharose CL-4B. The isolation and characterization of the monoclonal antibody, 3A3, which specifically recognizes rat pheochromocytoma (PC12) cell collagen/laminin receptor and perturbs PC12 cell adhesion to collagen and laminin, have been described elsewhere [44, 45].

Polyacrylamide gel electrophoresis and immunoblotting. Electrophoresis was performed on SDS–polyacrylamide gradient gels according to Blobel and Dobberstein [46]. Molecular weight standards were from Bio-Rad Laboratories. Reduced samples were treated with 10 m*M* DTT followed by alkylation with 50 m*M* iodoacetamide. Immunoblot analysis of proteins transferred to nitrocellulose was performed according to Gerton *et al.* [47]. The molecular weights of the blotted proteins were estimated by relating their positions to ^{125}I-labeled marker proteins (Sigma MW-SDS-200 marker protein kit supplemented with fibronectin) transferred from the SDS–polyacrylamide gel.

Peptide analysis. Peptide mapping was performed essentially as described by Cleveland *et al.* [48]. Hepatocyte proteins eluted by EDTA from collagen type I–Sepharose were dialyzed against PBS and iodinated using IODO-BEADS (Pierce Chemicals Co.) according to the recommendations of the manufacturer. The iodinated proteins were separated on 5–10% SDS–PAGE under reducing conditions. The gel was fixed and dried in solutions devoid of acid and subjected to autoradiography. The two high-M_r bands were cut out separately using the X-ray film as a template and stored in the freezer until used. Cleavages with trypsin, α-chymotrypsin, and V8-protease were performed in the spacer gel with subsequent separation on a 10–15% SDS–polyacrylamide gel. After completed electrophoresis the gels were dried and subjected to autoradiography.

Cell surface labeling and preparation of cell extracts. Confluent fibroblasts or overnight cultures of hepatocytes were labeled with ^{125}I in culture dishes (100 mm in diameter). The plates were incubated with 0.5 mCi ^{125}I (Amersham, UK), 50 μg of lactoperoxidase, 2 μg glucose oxidase all in 1 ml of PBS with 5 m*M* β-D-glucose for 20 min at room temperature. After the plates were washed with cold PBS (5× 10 ml), 1 ml of lysis buffer (1% Triton X-100 (TX-100), 1 m*M* $MnCl_2$, and 10 m*M* Tris–HCl, pH 7.4, containing 1 m*M* PMSF, 10 mg/ml aprotinin, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin) was added to each plate and the plates were then incubated for 30 min at 4°C. The cell layer was scraped and transferred to a test tube, insoluble mate-

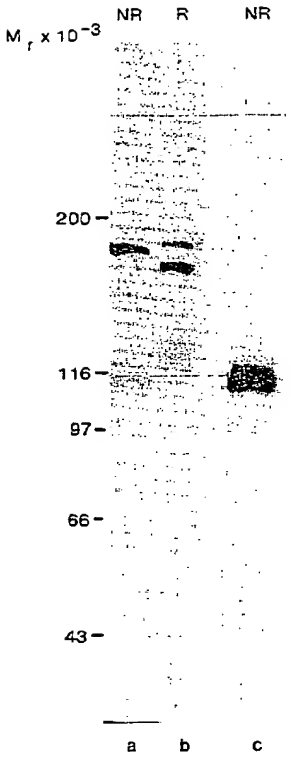

FIG. 1. Analysis of hepatocyte integrins with affinity for collagen type I. Hepatocyte proteins, purified on collagen type I–Sepharose and eluted with 10 m*M* EDTA as described under Materials and Methods, were electrophoresed on 5–10% SDS–polyacrylamide gels under nonreducing (NR; lane a) or reducing (R; lane b) conditions and visualized by silver staining. In lane c, EDTA eluted proteins that had been separated on a 5–10% SDS–gel under nonreducing conditions were transferred to nitrocellulose and immunoblotted with anti-β_1-integrin IgG.

rial was removed by centrifugation at 15,000*g* for 30 min, and NaCl was added to a final concentration of 0.13 *M*.

Immunoprecipitation. For immunoprecipitations with polyclonal rabbit IgG, cell extracts (or iodinated proteins affinity purified on collagen–Sepharose) were incubated with purified preimmune rabbit IgG (100 μg/ml) end over end for 4 h after which 100 μl of a suspension of Protein A–Sepharose CL-4B beads (50% slurry with 0.1% TX-100, 0.15 *M* NaCl, 10 m*M* Tris–HCl, pH 7.4) was added and allowed to bind for 1 h. The beads were removed by centrifugation and the cleared extract was incubated with the immune IgG (100 μg/ml) for 8 h, followed by a further 1-h incubation in the presence of 100 μl of Protein A–Sepharose slurry. For immunoprecipitations with monoclonal antibody 3A3 the same procedure was used except that the IgG was used at a concentration of 10 μg/ml, and antibodies were precipitated by goat anti-mouse IgG–Sepharose. After the last incubation the beads were washed four times with 1% TX-100, 0.5 *M* NaCl, 1 m*M* $MnCl_2$, 10 m*M* Tris–HCl, pH 7.4, and twice with TBS, prior to solubilization and boiling in SDS–PAGE sample buffer. All steps were performed at 4°C. After completed electrophoresis the gels were dried and exposed to Kodak X-Omat AR film.

Affinity chromatography. All steps were performed at 4°C. *Hepatocytes* were solubilized in lysis buffer (see above) with 0.13 *M* NaCl at 4°C for 1 h, the lysate was centrifuged at 100,000 *g* for 1 h, and the supernatant was applied to a lentil lectin column. The lectin column was washed in 0.1% TX-100, 0.5 *M* NaCl, 1 m*M* $MnCl_2$, 1 m*M* PMSF, and 10 m*M* Tris–HCl, pH 7.4 (Buffer A), followed by 0.1% TX-100, 1 m*M* $MnCl_2$, 1 m*M* PMSF, and 10 m*M* Tris–HCl, pH 7.4 (Buffer B), and eluted with Buffer B containing 5% α-methylmannoside. The lentil lectin eluate was then directly pumped onto a column of collagen–Sepharose or CB3–Sepharose, which was washed with Buffer B and Buffer B containing 75 m*M* NaCl, before eluting with elution buffer (0.1% TX-100, 10 m*M* EDTA, 10 m*M* Tris–HCl, pH 7.4). Eluted fractions were electrophoresed on SDS–polyacrylamide gels and proteins were visualized by silver staining. ^{125}I-labeled *fibroblasts* were extracted in lysis buffer and the lysate was incubated with collagen type I–Sepharose, CB3–Sepharose, or 105-kDa fibronectin fragment–Sepharose for 30 min. The columns were extensively washed with Buffer B and thereafter with Buffer B containing 75 m*M* NaCl, before eluting with elution buffer. Eluted proteins were visualized by autoradiography.

Cell attachment assay. The quantitation of cell attachment and the effects of various antibodies on this attachment have been previously described [15, 43]. Briefly, a microtiter plate-based assay was used in which proteins were coated on Microelisa plates (Dynatech, In vitro, Chantilly, VA), overnight at 4°C in the following concentrations: laminin, 20 μg/ml; calf collagen type I, 50 μg/ml; and fibronectin, 20 μg/ml. All proteins were dissolved in cold PBS. Remaining protein binding sites on the plastic surface were blocked by incubation with 0.1% BSA in PBS. In 100 μl of a buffer containing 0.137 *M* NaCl, 4.7 m*M* KCl, 0.65 m*M* $MgCl_2$, 1.2 m*M* $CaCl_2$, 10.1 m*M* Hepes, at pH 7.4, 10^4 cardiac fibroblasts or hepatocytes were seeded

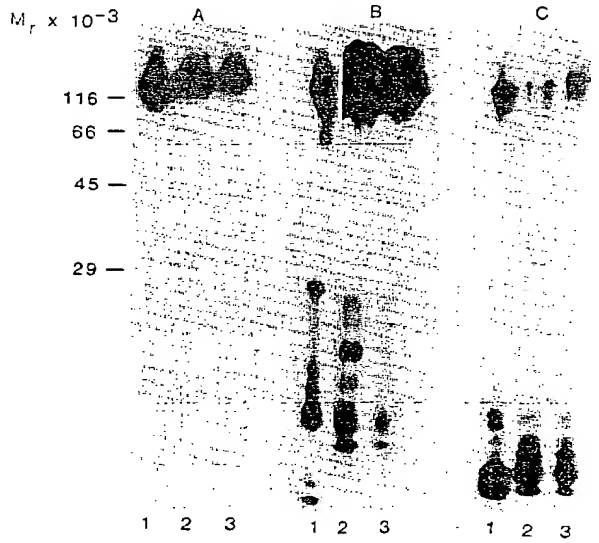

FIG. 2. Peptide mapping of the two protein bands comprising the hepatocyte collagen-binding integrin. The hepatocyte integrin with affinity for collagen was iodinated and electrophoresed on a 5–10% SDS gel under reducing conditions. Following drying and autoradiography the three bands shown in Fig. 1, lane b, were excised from the gel and subjected to cleavage in the spacer gel of a 10–15% SDS gel as described under Materials and Methods: β_1-integrin band (1); *M*, 180,000 band (2); *M*, 185,000 band (3). (A) uncleaved proteins; (B) cleavage with 1.0 μg chymotrypsin; (C) cleavage with 1.0 μg of V8 protease. Note the close resemblance of the fragment patterns in lanes 2 and 3.

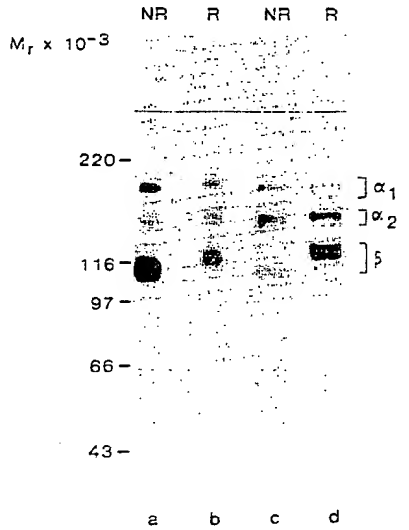

FIG. 3. Analysis of fibroblast integrins with affinity for collagen type I. Surface-iodinated rat heart fibroblast proteins were purified on collagen type I–Sepharose, eluted with 10 m*M* EDTA as described under Materials and Methods, and electrophoresed on a 5–10% SDS gel under nonreducing (NR; lane a) and reducing (R; lane b) conditions after which the gel was dried and labeled proteins were visualized by autoradiography. The eluted proteins were passed over a G-25 Sephadex column equilibrated in 0.1% TX-100, 1 m*M* $MnCl_2$, 0.15 *M* NaCl, 10 m*M* Tris–HCl, pH 7.4, and subjected to immunoprecipitation with anti-β_1-integrin IgG as described under Materials and Methods. The specifically precipitated proteins were separated on a 5–10% SDS gel under nonreducing (NR; lane c) and reducing (R; lane d) conditions followed by autoradiography.

in the presence of the indicated concentrations of the IgG to be tested. Cells were incubated for 30 min at 37°C, the plates were washed, and the numbers of attached cells were quantified as described [15].

Immunofluorescence. Reflection interference microscopy (RIM) images were achieved using the Bio-Rad MRC-600 confocal microscope in the reflectance mode. Cells grown on collagen type I- or fibronectin-coated glass coverslips were processed for RIM, immunofluorescence staining, and laser scanning confocal microscopy as previously described [15, 38]. Neonatal cardiac fibroblasts or hepatocytes were cultured on collagen type I- or fibronectin-coated coverslips overnight. Coverslips were rinsed in PBS and cells were fixed with 2% paraformaldehyde in PBS for 18 min (hepatocytes) or for 10 min (fibroblasts), rinsed in fresh PBS, permeabilized with 0.5% Triton X-100 in PBS for 20 min (hepatocytes) or for 30 min (fibroblasts), and incubated with 0.1 *M* glycine, pH 7.4, for 1 h followed by 0.5% BSA in PBS (PBS–BSA) for 30 min. Four different staining protocols were used: The 3A3 antibody (5–10 μg/ml), the anti-β_1-integrin IgG (40 μg/ml), and control IgG were incubated with fixed and permeabilized fibroblasts at 37°C for 45 min. The coverslips were rinsed with PBS–BSA and in *protocol 1* incubated with biotinylated anti-mouse or anti-rabbit IgG at 37°C for 30 min, rinsed with PBS–BSA, incubated with Texas Red-labeled avidin at 37°C for 30 min, and rinsed in PBS–BSA. Following a final rinse in distilled water the coverslips were mounted in Fluoromount-G. In *protocol 2*, the fixed and permeabilized fibroblasts were incubated with primary antibodies as above and after the cells were rinsed they were incubated with anti-mouse IgG conjugated with FITC, rinsed, and mounted [49]. In *protocol 3*, the fixed and permeabilized hepatocytes were incubated with anti-β_1 integrin IgG (40 μg/ml) and with control IgG at 37°C for 45 min. The coverslips were rinsed in PBS–BSA, incubated with anti-rabbit IgG conjugated with FITC at 37°C for 30 min, rinsed in PBS–BSA, and mounted. In *protocol 4*, fixed and permeabilized hepatocytes were incubated with 3A3 (5–10 μg/ml) at 37°C for 30 min. The coverslips were rinsed in PBS–BSA, incubated with rabbit anti-mouse IgG, rinsed in PBS–BSA, incubated with anti-rabbit IgG conjugated with FITC at 37°C for 30 min, rinsed, and mounted.

RESULTS

Isolation of Hepatocyte Integrin Receptors by EDTA Elution from Collagen Type I–Sepharose

Isolated rat hepatocytes were solubilized by 1% TX-100 in the presence of 1 m*M* $MnCl_2$. Solubilized proteins were sequentially purified on lentil lectin–Sepharose and collagen type I–Sepharose at low ionic strength. Elution from the latter column with 10 m*M* EDTA yielded two major proteins with M_r's under nonreducing conditions of 115,000 and 180,000 as analyzed by SDS–polyacrylamide gel electrophoresis

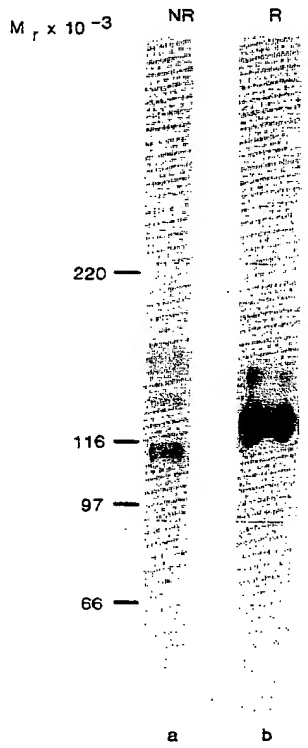

FIG. 4. Analysis of fibroblast integrins with affinity for fibronectin. Surface-iodinated rat heart fibroblast membrane proteins were purified on a column of fibronectin fragment–Sepharose and eluted with 10 m*M* EDTA as described under Materials and Methods. Eluted proteins were electrophoresed on a 5–10% SDS gel under nonreduced (NR; lane a) and reduced (R; lane b) conditions followed by autoradiography.

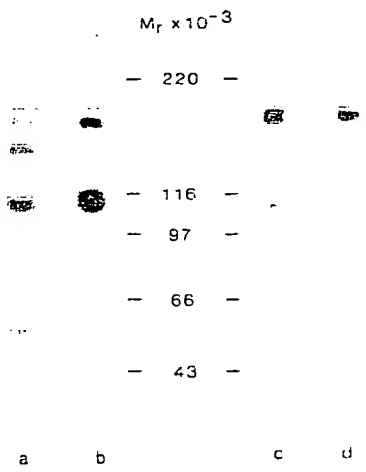

FIG. 5. Reactivity of the 3A3 Mab with a collagen-binding integrin of hepatocytes and fibroblasts. Surface-iodinated fibroblast integrins with affinity for collagen, and collagen-binding hepatocyte integrins that had been iodinated after affinity purification, were subjected to immunoprecipitation with the 3A3 monoclonal antibody. The collagen-binding integrins from fibroblasts were precipitated with anti-β_1-integrin IgG (lane a) and 3A3 IgG (lane b). Collagen-binding integrins from hepatocytes were immunoprecipitated with the rabbit polyclonal, anti-180-kDa IgG (lane c) and 3A3 IgG (lane d). The precipitated proteins were separated on a 5–10% SDS polyacrylamide gel under nonreducing conditions followed by autoradiography.

(SDS–PAGE) (Fig. 1, lane a). After reduction the eluted proteins migrated as three major bands, with M_r's 130,000, 180,000, and 185,000 (Fig. 1, lane b). Analysis by immunoblotting using anti-β_1-integrin IgG revealed the 115,000 protein to be a β_1-integrin (Fig. 1, lane c). Partial proteolytic digestion and analysis of the proteolysis products by SDS–PAGE showed that the two high M_r bands, present after reduction of the hepatocyte-derived proteins, yielded very similar peptide maps (Fig. 2).

Isolation of Fibroblast Integrin Receptors by EDTA Elution from Collagen Type I–Sepharose

Surface-iodinated rat cardiac fibroblasts were solubilized by 1% TX-100 on the culture plate in a buffer containing 1 m*M* $MnCl_2$ and the detergent-solubilized proteins were subsequently applied to columns of collagen type I-Sepharose or fibronectin fragment–Sepharose, respectively. Specific elution was achieved with 10 m*M* EDTA. The eluted proteins were analyzed by SDS–PAGE under reducing and nonreducing conditions (Figs. 3 and 4). Three major radioactive bands were eluted from the collagen type I–Sepharose with EDTA. The fastest migrating band of these appears to correspond in molecular weight to the integrin β_1-chain (M_r's 115,000 and 130,000 under nonreducing and reducing conditions, respectively). The middle band (M_r 145,000 nonreduced, tentatively designated α_2) and the upper band have the characteristics of integrin α-chains, suggesting that elution from collagen–Sepharose yielded a mixture of two integrin heterodimers (Fig. 3). When the proteins eluted from collagen–Sepharose by EDTA were immunoprecipitated with the anti-β_1-integrin IgG, only the larger (M_r 180,000 nonreduced, designated α_1) of the two putative α-chains was immunoprecipitated in association with the β_1-integrin band (data not shown). When, however, EDTA was removed by gel filtration prior to immunoprecipitation, all three protein bands were precipitated (Fig. 3, lanes c and d), confirming that the eluted proteins were a mixture of two β_1-integrins. The material eluted from fibronectin fragment–Sepharose showed, in addition to a protein corresponding in molecular weight and behavior after reduction to the integrin β_1-chain, potential integrin α-chains with M_r's of 130,000 and 145,000, respectively. The potential α-chains eluted from the fibronectin fragment–Sepharose appeared to migrate with lower M_r's after reduction

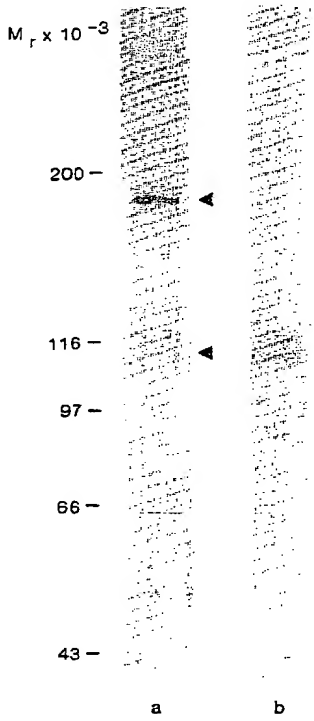

FIG. 6. Analysis of α1(I) CB3-binding integrins from hepatocytes. Hepatocyte proteins, purified on CB3–Sepharose and eluted with 10 m*M* EDTA as described under Materials and Methods, were electrophoresed on 5–10% SDS–polyacrylamide gels under nonreducing (NR; lane a) conditions and visualized by silver staining. A corresponding sample was electrophoresed under the same conditions and immunoblotted with anti-β_1-integrin IgG followed by autoradiography (lane b). Arrowheads denote positions of stained bands.

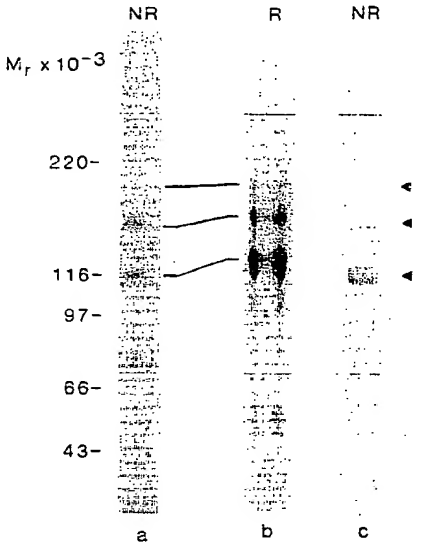

FIG. 7. Analysis of α1(I) CB3 binding on integrins from fibroblasts. Surface-iodinated rat heart fibroblast proteins were purified on CB3–Sepharose, eluted with 10 m*M* EDTA as described under Materials and Methods, and electrophoresed on a 5–10% SDS–polyacrylamide gel under nonreducing (lane a) or reducing (lane b) conditions followed by autoradiography. The eluted proteins were passed over a G-25 Sephadex column equilibrated in 0.1% TX-100, 1 m*M* $MnCl_2$, 0.15 *M* NaCl, 10 m*M* Tris–HCl, pH 7.4, and subjected to immunoprecipitation with anti-β_1-integrin IgG. The specifically precipitated proteins were separated on a 5–10% SDS–polyacrylamide gel under nonreduced conditions followed by autoradiography (lane c). Arrowheads in lane c show positions of weak bands that were seen on original autoradiogram after prolonged exposure.

(Fig. 4), a characteristic of α-chains of the human fibronectin integrin receptors, VLA-3 and VLA-5 [2, 5], and different from what was observed for the putative α-chains eluted from the collagen–Sepharose (Fig. 3).

Nature of the Isolated Integrin α-Chains

A recently described monoclonal antibody, designated 3A3, precipitates an integrin heterodimer (approximate M_r's 185,000 and 125,000) from rat pheochromocytoma (PC12) cells [44]. The 3A3 monoclonal antibody can be used to identify, or inhibit the function of, rat $\alpha_1\beta_1$-integrins homologous to the human VLA-1 [45].

Rabbit polyclonal antibodies were raised against the M_r 180,000 protein purified from rat hepatocytes. These antibodies specifically immunoprecipitated the 180,000/115,000 complex both from surface-iodinated hepatocytes and fibroblasts (data not shown). Integrin heterodimers, isolated from surface-iodinated cardiac fibroblasts by EDTA elution after affinity chromatography on collagen type I–Sepharose, were immunoprecipitated with either anti-β_1-integrin IgG or 3A3 IgG (Fig. 5). The 3A3 antibody, specific for the rat α_1-chain of the β_1-integrin subfamily, precipitated two labeled proteins, with M_r's (nonreduced) of 115,000 and 180,000 (Fig. 5, lane b). The anti-β_1-integrin IgG precipitated three bands, with M_r's (nonreduced) of 115,000, 145,000, and 180,000 (Fig. 5, lane a). In a similar experiment with hepatocyte-derived proteins iodinated after affinity chromatography, the polyclonal anti-180-kDa IgG precipitated a single (M_r 180,000/115,000) heterodimer (Fig. 5, lane c), while the 3A3 IgG precipitated only one major radioactive band, with an M_r of 180,000 (Fig. 5, lane d). The reason for the low yield of β-chain after immunoprecipitation reflects the lower amount of this chain present in the starting material used for iodination. These results confirm that the M_r 180,000 α-chains of both hepatocytes and fibroblasts are very similar and also establish that they are α_1-chains, i.e., rat homologs of the α_1-chain found in human VLA-1.

RGD Independency of the Binding to Collagen

We have previously described the binding of hepatocytes to the RGD-lacking CB3 α1(I) fragment of collagen type I [15, 28]. When hepatocyte cell extracts were purified by affinity chromatography on columns with

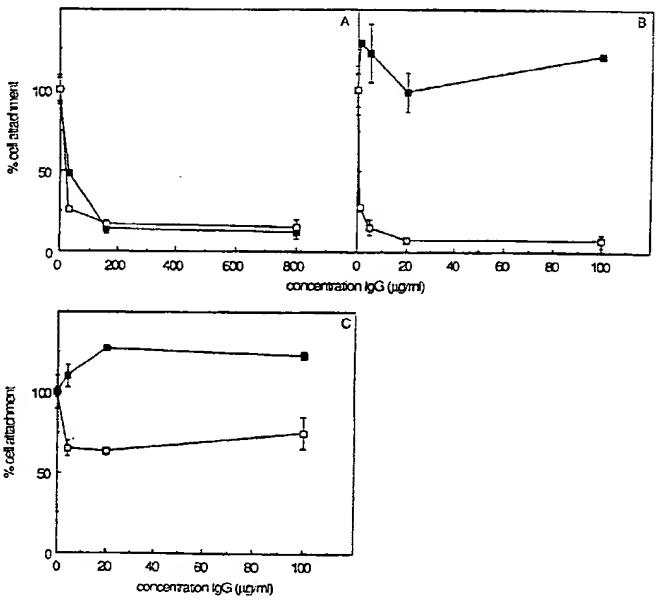

FIG. 8. Effects on cell attachment of the 3A3 monoclonal antibody. Hepatocytes (A and B) or rat cardiac fibroblasts (C) were seeded in 96-well microelisa plates coated with collagen type I (open symbols) or fibronectin (closed symbols) in the presence of indicated amounts of anti-β_1-integrin IgG (A) or 3A3 IgG (B and C). Plates were incubated for 45 min at 37°C and nonadherent cells were then washed off. Attachment is expressed as a percentage, with 100% cell attachment set as the attachment obtained without any IgG present. Vertical bars indicate the range of duplicate incubations.

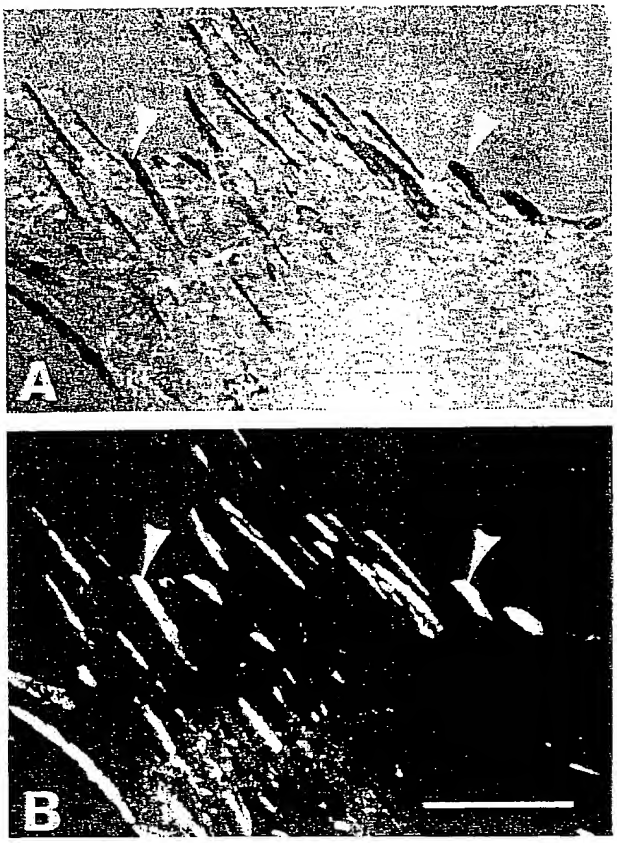

FIG. 9. Distribution of the rat VLA-1 homolog in focal contacts as revealed by RIM and immunofluorescent staining for the α-chain of the rat VLA-1 homolog. Neonatal cardiac fibroblasts were grown on thin native collagen films and focal contacts were visualized by RIM (A). Following fixation and permeabilization subsequent immunofluorescence staining according to protocol 2 described under Materials and Methods showed the distribution of the rat VLA-1 homolog (B). Bar in panel B represents 10 μm.

CB3 α1(I) coupled to Sepharose, EDTA eluted the same proteins as those from collagen type I columns according to SDS–PAGE analysis (Fig. 6), but with substantially lower yields. Two major protein bands with M_r's 180,000 and 115,000 (nonreduced) were evident in the silver-stained gels (Fig. 6, lane a); the lower of these was shown by immunoblotting to be the rat β_1-integrin chain (Fig. 6, lane b). When membrane proteins from surface-iodinated fibroblasts were similarly subjected to affinity chromatography on CB3–Sepharose, only very low amounts of radioactivity were eluted. After prolonged exposure of the autoradiogram three bands with M_r's of 115,000 145,000, and 180,000 became visible (Fig. 7, lane c). When the eluted fibroblast proteins were reduced prior to electrophoresis the M_r 145,000 and 180,000 proteins showed the same characteristic increase in apparent M_r as that observed for the proteins eluted from collagen type I–Sepharose (Fig. 7, lane b). Immunoprecipitation with anti-β_1-integrin IgG after change to an EDTA-free buffer confirmed the integrin nature of these eluted proteins (Fig. 7, lane c).

Attempts to specifically elute membrane proteins from collagen–Sepharose with peptides containing the RGD sequence were unsuccessful. Both RGDT and RGES eluted comparable levels of proteins. RGDT peptides, however, did specifically elute the β_1-integrin chain in association with an M_r 145,000 putative α-chain from fibronectin fragment–Sepharose (data not shown).

Function of the Rat VLA-1 Homolog in Collagen Adhesion

The 3A3 monoclonal antibody effectively inhibited the adhesion of both rat primary hepatocytes and cardiac fibroblasts to collagen type I-coated plastic surfaces (Figs. 8B and C). The efficiency of this inhibition of cell adhesion differed, however, for the two types of cells. The adhesion of hepatocytes to collagen type I-coated surfaces was completely inhibited, whereas the adhesion of fibroblasts to the same substrate could not be inhibited to more than around 40% (Figs. 8B and C). The adhesion of both cell types to fibronectin-coated surfaces was not affected by concentrations more than 10 times the inhibitory concentration on collagen type I-coated surfaces of the 3A3 antibody (Figs. 8B and C). The 3A3 antibody also inhibited the adhesion of cells to laminin-coated substrates in a manner similar to that observed for collagen type I-coated substrates; i.e., it completely inhibited the adhesion of hepatocytes but only partly the adhesion of fibroblasts to surfaces coated with this protein (data not shown).

Distribution of the Rat Homolog to VLA-1 in Cultured Cardiac Fibroblasts and Hepatocytes

The detection of focal adhesions by RIM, and immunofluorescence staining with the 3A3 monoclonal antibody on individual cells, revealed that the rat homolog to VLA-1 was present in focal adhesions on cardiac fibroblasts grown on native collagen films (Figs. 9A and B). This distribution of the α_1-chain in focal adhesions on cardiac fibroblasts was not observed when the cells were grown on fibronectin (Fig. 10D). When stained with a rabbit polyclonal anti-β_1-integrin IgG the cardiac fibroblasts exhibited focal adhesion-like staining patterns irrespective of the type of substrate on which the cells were grown (Figs. 10A and C). A similar relation between immunofluorescence staining patterns with the 3A3 monoclonal antibody was observed for hepatocytes that had been allowed to spread. Thus, when hepatocytes were cultivated on native collagen films and stained by the 3A3 monoclonal antibody, a discrete but

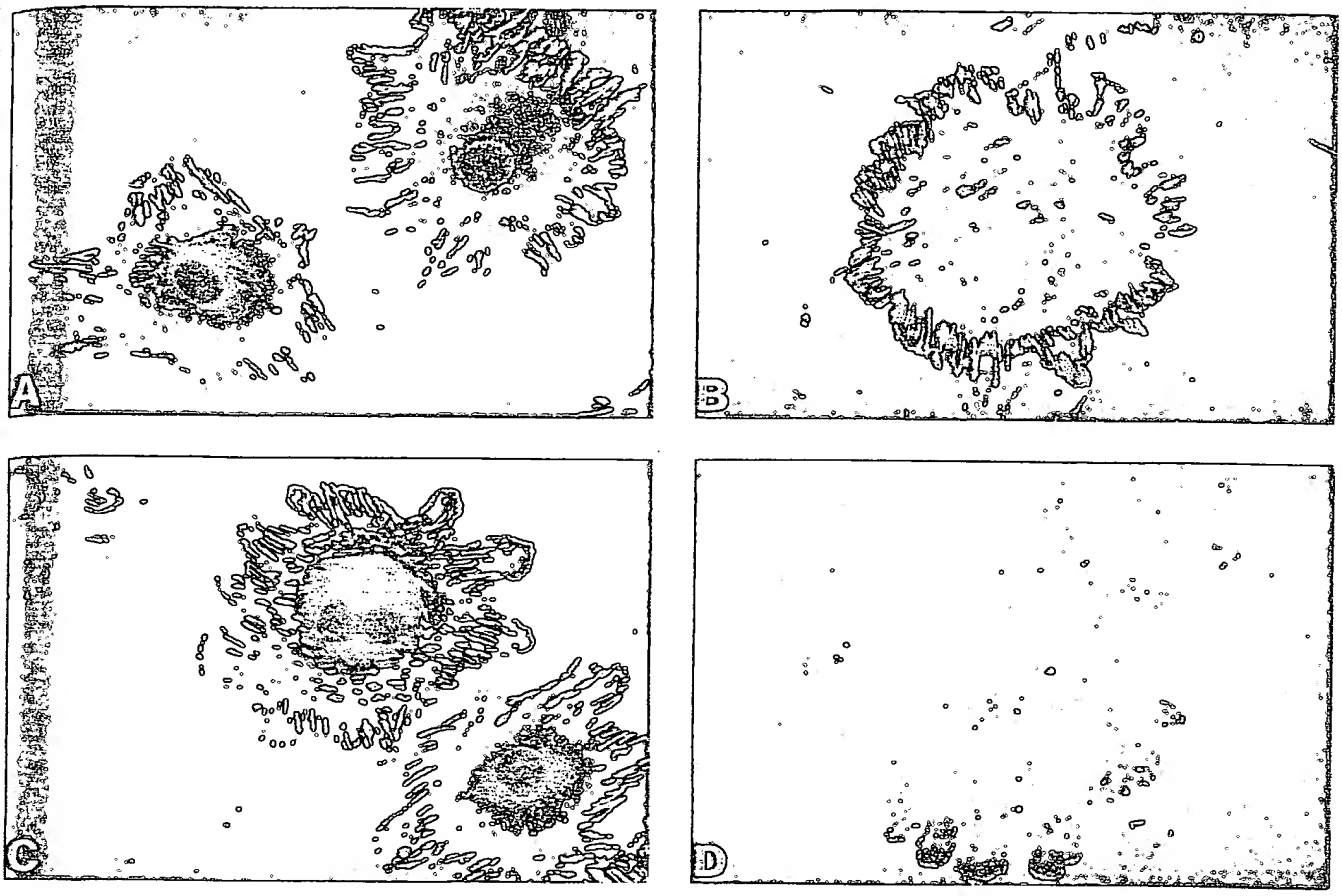

FIG. 10. Distribution of the rat β_1-integrin chain and the α_1-chain of the rat VLA-1 homolog in fibroblasts grown on collagen- and fibronectin-coated substrates. Neonatal rat cardiac fibroblasts were grown on coverslips coated with a thin film of native collagen (A and B) or with fibronectin (C and D). Cells were fixed, permeabilized, and subjected to immunofluorescence staining with anti-β_1-integrin IgG (A and C) or with 3A3 IgG (B and D). Staining was performed according to protocol 1 described under Materials and Methods. Magnification ×315.

weak patchy immunofluorescent staining, localized to the cell margins, was observed (Fig. 11B). The latter staining pattern was not observed when the cells had been cultivated on fibronectin-coated dishes (Fig. 11D). The rabbit polyclonal anti-β_1-integrin IgG stained hepatocytes cultivated on collagen or fibronectin mainly in a diffuse pattern at the central parts of the cells, as well as in a discrete patchy pattern at the cell circumferences (Figs. 11A and C). The latter pattern was qualitatively similar to the staining pattern observed for the 3A3 monoclonal antibody on hepatocytes grown on native collagen films (Figs. 11A and B).

DISCUSSION

In this report we have studied collagen-binding integrin heterodimers, isolated by affinity chromatography on collagen type I–Sepharose in the presence of Mn^{2+} and elution of bound proteins with EDTA [cf. 11, 50]. A single collagen-binding β_1-integrin with an M_r 180,000 α-chain was isolated from primary rat hepatocytes. By contrast, two species of collagen-binding β_1-integrins with α-chains having M_r's of 180,000 and 145,000, respectively, were isolated from primary rat cardiac fibroblasts. These rat collagen-binding integrins share biochemical characteristics with the human VLA-1 and VLA-2 integrin heterodimers, respectively, both of which are known to bind collagen [14, 44, 51].

The identity of the M_r 180,000 α-chain was investigated further using a monoclonal antibody, designated 3A3, that specifically recognizes the M_r 185,000 α-chain of a rat β_1-integrin isolated from rat pheochromocytoma (PC12) cells [44]. A partial N-terminal sequence for the α-chain of the rat pheochromocytoma integrin recognized by the 3A3 antibody is identical at 11 of 13 positions with that for the α_1-chain found in the human β_1-integrin designated VLA-1 [45]. The finding that the rat hepatocyte and cardiac fibroblast M_r 180,000 α-chains were immunoprecipitated by 3A3 IgG strongly suggests

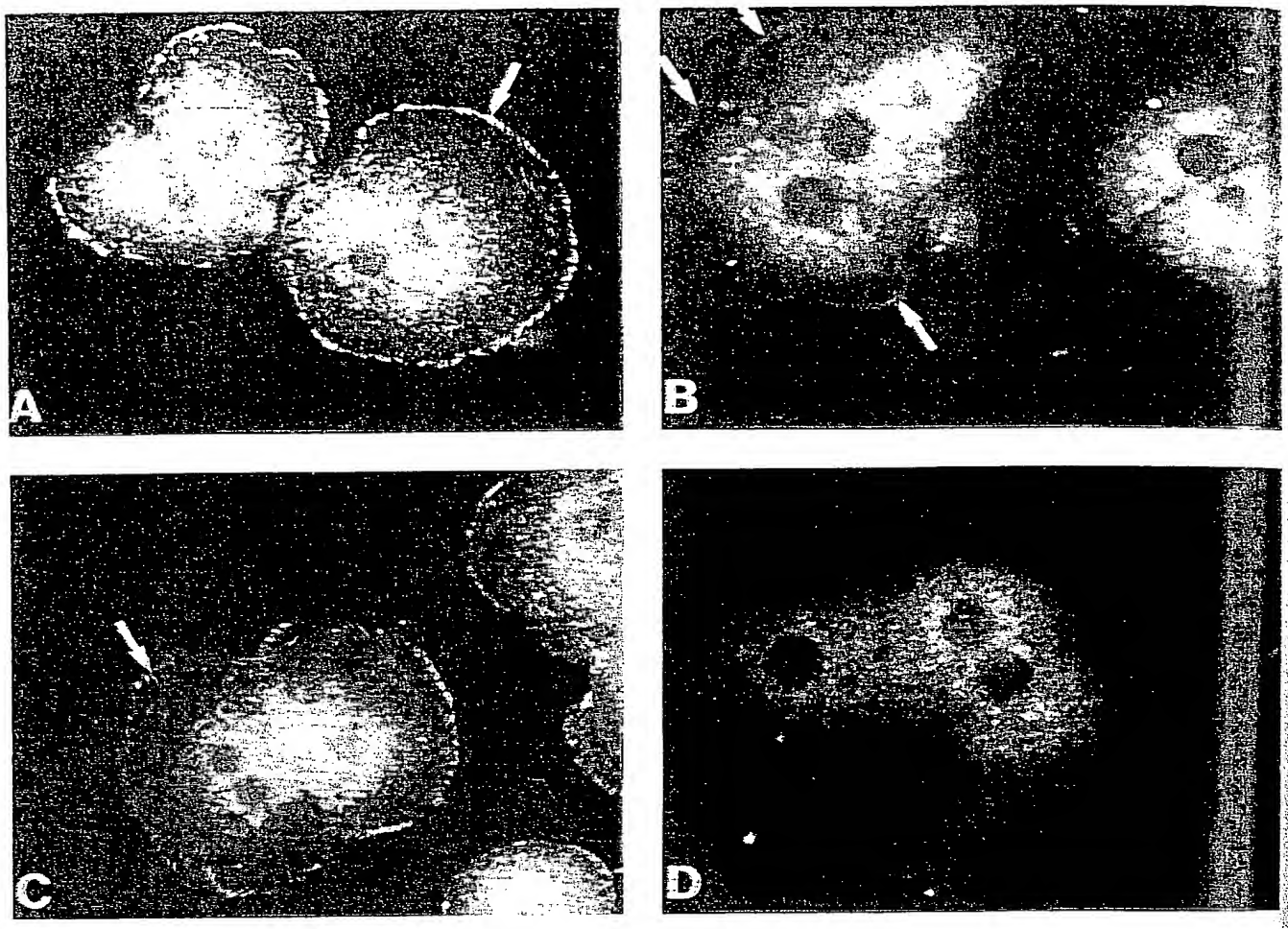

FIG. 11. Distribution of the rat β_1-integrin chain and the α_1-chain of the rat VLA-1 homolog in hepatocytes grown on collagen- and fibronectin-coated substrates. Adult rat hepatocytes were grown on coverslips coated with a thin film of native collagen (A and B) or with fibronectin (C and D). Cells were fixed, permeabilized, and subjected to immunofluorescence staining with anti-β_1-integrin IgG (A and C) or with 3A3 IgG (B and D). Staining was performed according to protocols 3 and 4 described under Materials and Methods. Arrows indicate positive staining at the circumference of the hepatocytes. Magnification ×560.

that the rat M_r 180,000 β_1-integrin heterodimer is the rat homolog of the human VLA-1 integrin heterodimer. Upon reduction, the α_1-chains of the hepatocyte collagen receptor migrate as a closely spaced doublet (Fig. 1). Because the two bands have very similar peptide maps (Fig. 2), this heterogeneity almost certainly reflects a splitting, due to partial proteolytic degradation or other partial post-translational modification, of a single α_1-band. This feature was not seen with α_1-chains from surface-iodinated fibroblasts or PC12 cells [44], and thus is tissue- rather than species-specific. Its physiological relevance, if any, is unknown.

The monoclonal 3A3 antibody, which inhibits the adhesion of rat pheochromocytoma (PC12) cells both to collagen type I and laminin [44], completely inhibited the adhesion of rat hepatocytes to collagen. This finding is in good agreement with the biochemical evidence for a single hepatocyte integrin collagen receptor with an α_1-chain recognized by the 3A3 antibody. In contrast, the 3A3 antibody only partly inhibited the adhesion of fibroblasts to collagen. The latter finding, indicating the presence of an additional collagen receptor present on the fibroblasts, is likewise in concordance with the biochemical evidence presented above. The 3A3 antibody also specifically stained focal contacts formed by fibroblasts grown on collagen but not on fibronectin. A similar specificity for adhesive structures formed on collagen was observed for hepatocyte–substratum contacts. Thus, on the basis of biochemical and functional data presented in the present study we suggest that, in the liver, the VLA-1 heterodimer fulfills the role of anchoring the hepatocytes to collagens.

The ability of two collagen-binding integrins described in this report to bind α1(I) CB3, a fragment of collagen type I that lacks RGD sequences, as well as the inability of RGD-containing synthetic peptides to specifically elute the VLA-1 homolog and the M_r 145,000/β_1 integrin from collagen type I–Sepharose, corroborates our earlier functional studies demonstrating that the attachment to collagen of the two types of primary cells used in this study occurs via an RGD-independent mechanism [15]. Other β_1-integrins found to bind their ligands RGD-independently include laminin receptors [10–12], a fibronectin receptor on T-lymphocytes [52], and collagen receptors on human melanoma cells [14]. Our data also show that the CB3 α1(I) fragment contains cell binding sites for β_1-integrins. A comparison of the integrins that bind the α1(I) CB3 fragment shows that from hepatocytes the VLA-1 homolog clearly binds, whereas from fibroblasts there is relatively more of the M_r 145,000/β_1-integrin that binds α1(I) CB3 compared with the fibroblast VLA-1 homolog [cf. Figs. 6 and 7]. It is possible that this reflects cell-type-specific differences in the affinity of these murine VLA-1 homologs for the α1(I) CB3 fragment. This possibility is currently being investigated.

Whereas rat hepatocytes appear to have a single collagen receptor (the VLA-1 homolog), fibroblasts have both the VLA-1 homolog and an integrin heterodimer that possess a putative α-chain with an M_r of 145,000. Conceivable explanations for the presence of two receptors with affinity for the same ligand are that they bind to separate sites in the ligand and/or that they have separate functions. It is plausible that the M_r 145,000/β_1 rat cardiac fibroblast integrin, which shares characteristics with the human VLA-2 heterodimer, is involved in cell migration. In support of such a hypothesis is a recent report showing that WI-38 fibroblasts utilize VLA-2 for migration on collagen [53]. The apparent absence of the M_r 145,000/β_1 collagen-binding heterodimer from the stationary hepatocytes would also support the concept of a migration-related function of this receptor. To delineate these possibilities a combination of functional integrin α-specific antibodies and different cyanogen bromide fragments of collagen can be used.

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The Integrin α1 A-domain Is a Ligand Binding Site for Collagens and Laminin*

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The integrin α1β1 is a cell surface receptor for collagens and laminin. The α1 subunit contains an A-domain, and the A-domains of other integrins are known to mediate ligand binding. To determine the role of the α1 A-domain in ligand binding and the extent to which it reproduced the ligand binding activity and specificity of the parent molecule, we produced recombinant α1 A-domain and tested its ability to bind collagens and laminin. In solid phase assays, the A-domain from α1 was found to bind to collagen I, collagen IV, and laminin in a largely cation-dependent manner. The α2 A-domain, from the α2β1 integrin, also bound to these ligands, but the binding hierarchy differed from that seen for α1. This is the first demonstration of laminin binding by A-domains. Specificity of A-domain-ligand binding was further investigated using the triple-helical proteolytic fragment of collagen IV, CB3, and its subfragments, F1 and F4. α1 A-domain bound to all three fragments, while the α2 A-domain bound CB3 less well and exhibited little binding to F1 and no binding to F4. These differences mirror previous reports of distinct integrin binding sites in collagen IV and for the first time identify a limited proteolytic fragment of a ligand that contains integrin A-domain binding activity. To gain insight into the contribution that the A-domain makes to ligand binding within the whole integrin heterodimer, we measured binding constants for A-domain-collagen interactions using surface plasmon resonance biosensor technology. The values obtained were similar to those reported for intact integrin binding, suggesting that the A-domain is the major collagen binding site in the α1β1 and α2β1 integrins.

Integrins are a family of αβ heterodimeric cell surface receptors, responsible for cell-cell and cell-extracellular matrix interactions. The specificity and regulation of these interactions is critical to many biological processes, including embryonic cell migration, wound healing, and the immune response (1). The integrin family contains at least 16 α subunits, seven of which contain an ~200 amino acid inserted domain in their N-terminal region (I or A-domain) (2, 3). This domain is homologous to the von Willebrand factor A-domain, a module also found in a number of other membrane, plasma, and matrix proteins (2, 4).

Mapping studies that have localized the epitopes of anti-functional monoclonal antibodies to the A-domains of the α1, α2, αL, αM, and αX integrins initially suggested a role for A-domains in ligand binding (5–10). More recently, it has also been demonstrated that isolated recombinant A-domains from α2, αL, and αM are capable of binding ligands (11–15).

The integrin α1β1 is a receptor for collagen I, collagen IV, and laminin (16). These interactions, like all integrin-ligand binding events, are cation-dependent and require Mg^{2+} or Mn^{2+}; Ca^{2+}, however, does not support binding (17). A similar pattern of ligand binding is found for the closely related integrin α2β1, which also interacts with collagen I, collagen IV, and laminin (16). α1β1 and α2β1 have, however, been shown to differ in their relative affinities for ligands, with α1β1 binding collagen IV and laminin with higher affinity (18). Integrins α1β1 and α2β1 also bind to a proteolytic fragment of collagen IV, CB3 (19), but are believed to have distinct binding sites within this fragment (18).

To investigate the role of the α1 A-domain in ligand binding, we have generated recombinant A-domain in a bacterial expression system and tested its binding to a range of integrin ligands. The specificity of A-domain ligand binding has also been examined by comparing recombinant α1, α2, and αM integrin A-domains. We find that integrin α1 A-domain binds to collagen I, collagen IV, and laminin in a saturable, concentration-dependent manner and that the binding can be inhibited by an anti-functional anti-α1 monoclonal antibody (mAb).[1] α2 A-domain also binds these ligands, while αM A-domain does not. The αM A-domain does, however, bind to fibrinogen, while the α1 and α2 A-domains do not. This binding is largely cation-dependent, and real time binding studies using surface plasmon resonance (SPR) analysis suggests that cation, A-domain, and ligand may form a ternary complex. By using proteolytic fragments of collagen IV, the ligand specificity of the closely related α1 and α2 A-domains has been compared. The results indicate that recombinant A-domains retain the specificity reported for intact receptors.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. Antibodies were obtained from the following sources: monoclonal mouse anti-human integrin α1, 5E8D9 (Upstate Biotechnology Inc., New York); monoclonal mouse anti-human

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[1] The abbreviations used are: mAb, monoclonal antibody; RT-PCR, reverse transcriptase-polymerase chain reaction; GST, glutathione *S*-transferase; TBS, tris-buffered saline; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; ABTS, 2′2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid).

integrin α2, Gi9 (The Binding Site, Birmingham, United Kingdom.) and 16B4 (previously reported as 1C11) raised as described by Mould *et al.* (20); monoclonal mouse anti-human integrin αM, 44 (Autogen Bioclear, Devizes, UK); anti-glutathione *S*-transferase (GST) rabbit polyclonal antibody (Autogen Bioclear). Rat tail tendon type I collagen, human placental type IV collagen, and human fibrinogen were obtained from Sigma, Poole, Dorset, UK. Rat laminin was obtained from Life Technologies Ltd., Paisley, UK. The collagen IV fragments CB3, F1, and F4 were produced as described previously (18, 19).

Production of Recombinant Integrin A-domains—The production of recombinant α2 A-domain has already been described (12). The α1 and αM A-domains were produced in a similar manner. DNA coding for the A-domains from integrins α1 and αM was produced by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA prepared from the A375 human melanoma cell line (for α1) or human buffy coat lymphocytes (for αM) was a gift of L. J. Green (University of Manchester, UK). First strand cDNA was generated using a 3′ primer spanning the predicted end of the integrin A-domains and incorporating a *Sal*I site (α1, 5′-TTTGTCGACTCAGGCTTCCAGG GCAAATATTCTTTCTC-C-3′; αM, 5′-TTTGTCGACTCAACCCTCGATCGCAAAGATCTTCTCC-CG-3′). Polymerase chain reaction (PCR) amplification of this cDNA was then carried out using the thermostable proof-reading DNA polymerase Pfu (Stratagene Ltd., Cambridge, UK) with the above 3′ primer and a 5′ primer designed to produce DNA coding for 17 amino acids preceding the predicted start of the integrin A-domain (21) and including a *Bam*HI site (α1, 5′-TTTGGATCCGTCAGCCCCACATTTCAAGT-CGTGAATTCC-3′; αM, 5′-TTTGGATCCAACCTACGGCAGCAGCCCC-AG-3′). 50 cycles, each consisting of 1 min at 94 °C, 1 min at 55 °C, and 2.5 min at 72 °C, were carried out. PCR products, of the correct M_r, were then excised from a 1% agarose gel. After digestion with *Bam*HI and *Sal*I, the products were ligated into pUC119 and used to transform *Escherichia coli* strain DH5αF′. A-domain sequences from transformants were sequenced by the dideoxy chain termination method of Sanger (22) and compared with the published sequences (α1 and αM, see Refs. 23 and 24, respectively). The sequenced DNA was then subcloned into the expression vector pGEX-4T3 (25) (Pharmacia, Milton Keynes, UK). Transformants were screened, and protein was produced as described for α2 (12) except that dithiothreitol was not required for GST removal from α1 and αM A-domains. For experiments using fusion protein, a single glutathione affinity step was performed and thrombin digestion was not carried out. The recombinant A-domains produced were 214 (α1), 224 (α2), and 211 (αM) amino acids long. N-terminal amino acid sequencing of thrombin-cleaved α1 and αM A-domains confirmed the predicted starting sequence.

Biotinylation of Proteins—A-domain-GST fusion proteins, collagen I, and fibrinogen were biotinylated as follows. Protein was diluted to 1 mg/ml in 150 mM NaCl, 10 mM Tris-HCl, pH 7.5 (TBS), and dry sulfo-*N*-hydroxysuccinimido-biotin (Pierce, Chester, UK) was added to give a ratio of 1:1 (w/w) protein:biotin. The mixture was incubated for 1 h at room temperature and then dialyzed against TBS (or 0.1 M acetic acid for collagen I) to remove unincorporated biotin.

Ligand Binding Assays—Binding of soluble ligand to immobilized A-domain was measured using assays adapted from Tuckwell *et al.* (12). A-domain fusion proteins (10 μg/ml in PBS without divalent cations, PBS−) were coated to 96-well microtitre plates (Immulon 4, Dynatech, Billingshurst, UK) overnight at 4 °C. Wells were then blocked with 50 mg/ml BSA in TBS for 1 h at room temperature and, washed twice with TBS, and biotinylated ligand in TBS, 1 mg/ml BSA, plus cation, was added for 3 h at 37 °C. Plates were washed three times with TBS, 1 mM $MnCl_2$, and ExtrAvidin peroxidase (Sigma; 10 μg/ml in TBS, 1 mM $MnCl_2$) was added for 15 min. After washing three times with TBS, 1 mM $MnCl_2$, bound ligand was visualized with 0.1 M sodium acetate, 0.05 M NaH_2PO_4, 2 mM 2′2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 0.03% H_2O_2. Wells were read at 405 nm on a plate reader.

A-domain Binding Assays—Assays measuring binding of soluble A-domains to immobilized ligand were carried out essentially as above, but plates were coated with ligand (usually at 30 μg/ml) instead of fusion protein and binding of biotinylated A-domain fusion protein was measured. For antibody detection of unbiotinylated A-domain fusion protein binding to ligand, the assay was performed as above up to the streptavidin step where 5 μg/ml anti-GST antibody in TBS, 1 mg/ml BSA, 1 mM $MnCl_2$ was added for 1 h instead. Wells were washed three times with TBS, 1 mM $MnCl_2$, and 1:500 peroxidase-linked goat anti-rabbit antibody (Sigma) in TBS, 1 mg/ml BSA, 1 mM $MnCl_2$ was added for 45 min. Wells were washed three times with TBS, 1 mM $MnCl_2$, and binding was detected with ABTS as above.

Surface Plasmon Resonance—The kinetic parameters (apparent association and dissociation rate constants k_a and k_d, respectively) and the apparent equilibrium constant (K_D) for A-domain binding to collagen I and IV were measured using SPR on a BIAcore™ (Biacore, St. Albans, UK). This biosensor device was used in accordance with the manufacturer instructions. Briefly, collagen I or IV was covalently coupled via primary amine groups to the dextran matrix of a CM5 sensor chip. A-domain solution was flowed over the chip at 5 μl/min, and binding was measured as a function of time. TBS, 1 mM $MnCl_2$ was used as running buffer throughout, and injections of TBS, 10 mM EDTA were used to remove bound A-domain, regenerating the surface for further binding experiments. The curve fitting software, BIAevaluation, was used to fit these results to the simple first order interaction model A + B ⇔ AB. This produced values for k_a and k_d, allowing the apparent equilibrium constant K_D to be derived from k_d/k_a. To measure binding in the presence of divalent cations other than Mn^{2+}, a 10-min injection of TBS, 10 mM EDTA was passed over the chip (to remove any residual Mn^{2+}) immediately prior to injection of A-domain in the specified cation.

RESULTS

Generation of Integrin α1 and αM A-domain cDNA—The reverse transcriptase-polymerase chain reaction was used to generate integrin α1 A-domain cDNA from A375 human melanoma cell line RNA and integrin αM A-domain cDNA from human buffy coat lymphocyte RNA. After cloning into pUC119, sequencing revealed that the αM A-domain cDNA matched the published sequence (24). Sequencing of α1 A-domain cDNA revealed three differences from the published sequence (23): 1) an inserted Thr at position 502; 2) a deleted Ala at position 511, which put the sequence back in frame after the earlier insertion, and 3) a Cys to Thr mutation at position 674. These differences result in two changes in the predicted amino acid sequence of the protein, a lysine to glutamate at position 170 and a threonine to isoleucine at 228, numbered from the start of the mature polypeptide. Repeating the RT-PCR using two different A375 RNA samples produced the same sequence, and RT-PCR using human smooth muscle RNA also gave the same sequence. As all PCR reactions used a proof-reading DNA polymerase and produced the same sequence from different RNA samples, we believe that it represents an accurate human α1 integrin sequence.

Expression of Integrin α1 and αM A-domains in E. coli—Integrin A-domain cDNAs were cloned into the pGEX-2T3 expression vector and used to transform *E. coli*. After induction, transformants expressed GST-A-domain fusion proteins of ~50 kDa as reported for the α2 A-domain (12). Fusion proteins were purified on glutathione-agarose columns and were either used directly or cleaved with thrombin and passed through a second glutathione-agarose column to produce purified 25 kDa A-domain. N-terminal sequencing of purified A-domains showed that cleavage had occurred at the expected site. SDS-PAGE showed the recombinant protein was at least 90% pure (data not shown).

All A-domains reacted specifically with previously characterized mAbs: the α2 A-domain bound a number of anti-α2 integrin mAbs, including Gi9, an anti-functional mAb. The α1 A-domain was recognized by the anti-functional anti-α1 integrin mAb 5E8D9 in ELISA, and αM A-domain bound the anti-functional anti-αM mAb 44 (data not shown).

Collagen I Binds to α1 and α2 A-domains but Not to αM A-domain—The integrins α1β1 and α2β1 are reported to be collagen receptors, while integrin αMβ2 binds non-collagenous ligands. To investigate the role of A-domains in binding collagen, the binding of biotinylated collagen I to A-domain fusion proteins was measured. Fig. 1 shows that the A-domain fusion proteins from α1 and α2 integrins support dose-dependent, saturable binding of collagen I, while the αM A-domain exhibits very little binding. Collagen I shows a higher maximal binding to the α1 A-domain than to the α2 A-domain. The observed differences in binding were not due to different coating efficien-

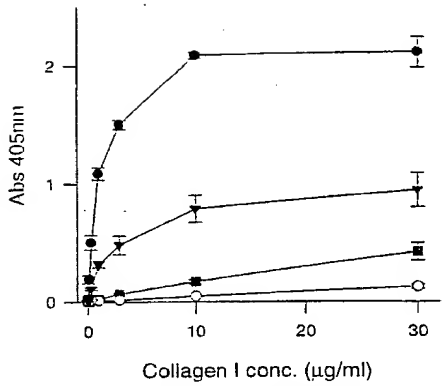

FIG. 1. **Collagen I binding to immobilized A-domains in ligand binding assays.** Binding to α1 A-domain fusion protein (●), α2 A-domain fusion protein (▼), αM A-domain fusion protein (■), and BSA only (○) in 1 mM $MnCl_2$ was measured. Data are mean ± S.E. and $n \geq 9$ from three experiments normalized for 30 μg/ml collagen binding to α1 A-domain.

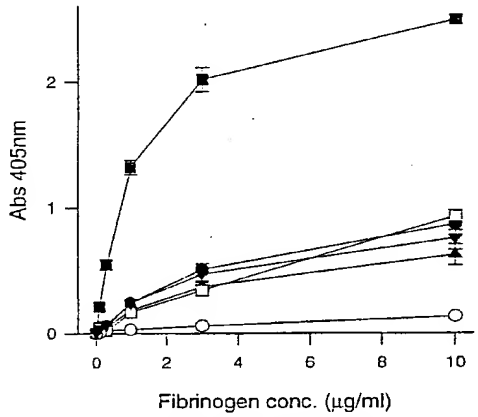

FIG. 2. **Fibrinogen binding to immobilized A-domains in ligand binding assays.** Binding to α1 A-domain fusion protein (●), α2 A-domain fusion protein (▼), αM A-domain fusion protein (■), GST (▲), and BSA (○) in 1 mM $MnCl_2$ and to αM A-domain in 5 mM EDTA (□) was measured. Data are mean ± S.E. and $n \geq 12$ from five experiments normalized for 10 μg/ml fibrinogen binding to αM A-domain.

cies of the GST-fusion proteins as the coating concentration chosen (10 μg/ml) gave very similar, almost maximal, coating of all three fusion proteins to the plate as measured by anti-GST antibody in ELISA (data not shown). The differences between α1 and α2 A-domains may instead reflect differences in the amount of correctly folded A-domain in the different samples rather than a difference in the number of binding sites per A-domain. Binding of collagen I was dependent on the conformation of the triple helix, as heat denaturation of the collagen I at 50 °C for 30 min inhibited its binding to α1 and α2 A-domains (data not shown).

Fibrinogen Binds the αM A-domain Specifically in a Cation-dependent Manner—To confirm the specificity of the A-domains for their ligands, we investigated binding of the known αMβ2 ligand fibrinogen to all three A-domains. Biotinylated fibrinogen bound to the αM A-domain in the presence of 1 mM Mn^{2+} but did not bind to α1 or α2 A-domains, above the GST control (Fig. 2). Interestingly the binding to GST was higher than to BSA. Fibrinogen binding to αM A-domain was reduced to GST levels by 5 mM EDTA indicating that the binding was cation-dependent. The binding of biotinylated fibrinogen to αM

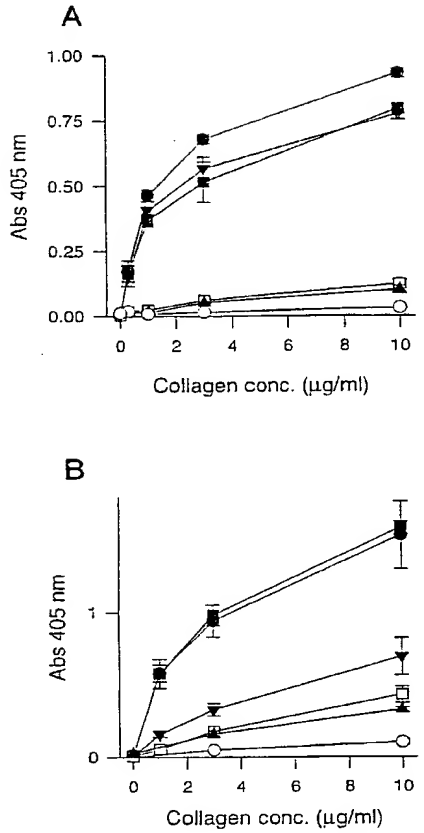

FIG. 3. **The effect of cations on collagen I binding to immobilized α1 (*A*) and α2 (*B*) A-domain fusion protein was measured in the presence of 1 mM $MnCl_2$ (●), 1 mM $MgCl_2$ (■), 1 mM $CaCl_2$ (▼), and 5 mM EDTA (▲).** Binding to GST (□) and BSA (○) was measured in 1 mM $MnCl_2$. Data are mean ± S.E. and $n \geq 9$ from three experiments normalized for 10 μg/ml collagen binding to A-domain in the presence of 1 mM $MnCl_2$.

A-domain was also reduced to the levels of the GST control by the anti-functional anti-αM mAb 44 (data not shown).

Cation-dependence of Collagen I Binding to A-domains—Having shown that the αM A-domain interaction with fibrinogen required divalent cations, we investigated the effect of different divalent cations on biotinylated collagen I binding to α1 and α2 A-domains. Fig. 3*A* shows that collagen binding to α1 A-domain was completely inhibited by EDTA and, therefore, requires divalent cations. However, the nature of the divalent cation had little effect as Ca^{2+}, Mg^{2+}, and Mn^{2+} all supported similar levels of binding. Of the three cations, Mn^{2+} supported the highest levels of binding. This cation profile does not match that reported for the whole integrin, where Mg^{2+} was required for ligand binding and Ca^{2+} did not support binding (17). Collagen binding to α2 A-domain was inhibited by EDTA (Fig. 3*B*); however, in this case Ca^{2+} supported only very little binding, and Mg^{2+} and Mn^{2+} produced identical levels of collagen binding. This is in agreement with previously published data (12). Thus, while the A-domains from integrins α1 and α2 both bind collagen I, they differ in their cation specificities.

The binding of soluble A-domain to immobilized collagen was also investigated. This was carried out using biotinylated A-domain fusion proteins or by antibody detection of the GST moiety on the A-domain fusion protein. Both methods demonstrated concentration-dependent saturable binding of α1 A-domain to collagen I; however, EDTA only partially inhibited this

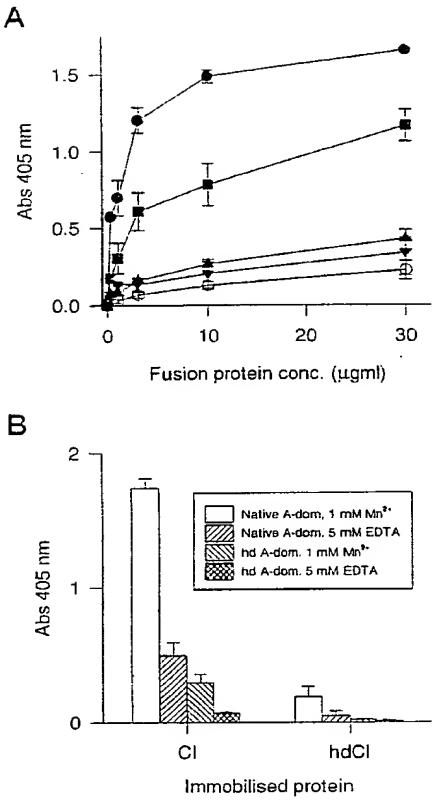

FIG. 4. **α1 A-domain binding to immobilized collagen I.** *A*, microtitre plates were coated with substrate at 30 μg/ml and blocked with BSA, and GST-A-domain fusion protein was added. After incubating for 3 h at 37 °C, unbound fusion protein was washed off. Binding was detected using anti-GST antibody, a peroxidase-conjugated goat anti-rabbit IgG secondary antibody, and ABTS. Binding of α1 A-domain fusion protein to collagen I in 1 mM $MnCl_2$ (●) and 5 mM EDTA (■) and to BSA in 1 mM $MnCl_2$ (○). Shown are the binding of GST in 1 mM $MnCl_2$ to collagen I (▲) and BSA (▼). Data are mean ± S.E. and $n \geq 9$ from four experiments normalized for 30 μg/ml α1 A-domain binding to collagen I. *B*, alternatively biotinlyated A-domain fusion protein was used, and binding was detected using ExtrAvidin-peroxidase and ABTS. The effect of heat denaturation of collagen I (*hdCI*), or α1 A-domain (*hd A-dom.*) by heating to 50 °C for 30 min, on ligand binding was investigated. Results are mean ± S.E. and $n = 9$ from three experiments. Background binding to BSA has been subtracted and results normalized for native α1 A-domain binding to native collagen I in the presence of 1 mM $MnCl_2$.

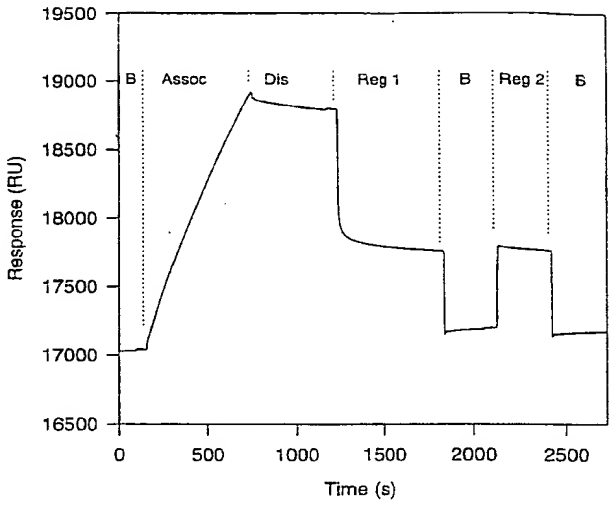

FIG. 5. **Sensorgram of α1 A-domain fusion protein binding to collagen I immobilized on a BIAcore CM5 sensor chip.** The sensorgram is annotated to indicate different stages in the experiment. Running buffer was TBS, 1 mM $MnCl_2$. *B* indicates that only running buffer was passing over the chip surface. *Assoc* shows binding to the chip during injection of 10 μg/ml α1 A-domain fusion protein. During the dissociation phase (*Dis*), only running buffer was again passing over the chip. Bound A-domain was removed during the regeneration phases (*Reg 1* and *Reg 2*). This involved injection of TBS, 10 mM EDTA. Following the initial regeneration, most of the binding was removed, and further injections of EDTA did not remove the residual binding. The large change in response during regeneration is due to differences in the refractive index of the buffers.

interaction (Fig. 4*A*). Inhibition was similar in both biotinylation and antibody detection assays, with slightly more inhibition seen for the antibody detection assays (data not shown). In both assays, inhibition was maximal at lower A-domain protein concentrations. Similar results were obtained for α2 A-domain binding to collagen I (data not shown).

The discrepancy between cation-dependence of collagen binding to immobilized A-domain and A-domain binding to immobilized collagen is difficult to explain. The interaction appeared to be specific as, under these conditions, binding was still dependent on the triple-helical conformation of collagen and the native A-domain structure, as heat denaturation of either component strongly inhibited binding (Fig. 4*B*, and data not shown).

To address the apparent discrepancies in solid phase assay results, the cation dependence of the A-domain-collagen interaction was further investigated using SPR measurements on a BIAcore. Collagen I was covalently immobilized onto the sensor chip, and binding of A-domain was measured. These measurements were performed for both α1 and α2 A-domain fusion proteins at a range of concentrations (3–100 μg/ml). Fig. 5 shows a typical sensorgram of α1 A-domain binding to collagen I in the presence of 1 mM Mn^{2+}. Association and dissociation phases are marked and removal of bound A-domain with 10 mM EDTA is shown. EDTA injection removed 86 ± 2% of the bound α1 A-domain (mean ± S.E.; $n = 33$) and 82 ± 8% of the bound α2 A-domain (mean ± S.E.; $n = 10$), indicating that the presence of divalent cation is required for maintenance of the bound complex, not simply for A-domain-collagen binding. GST failed to bind collagen in these assays. Binding of fusion proteins in the presence of different cations was also investigated, and, as already seen for biotinylated collagen binding to A-domains, 1 mM Mn^{2+}, Mg^{2+}, and Ca^{2+} all supported α1 A-domain binding while only Mn^{2+} and Mg^{2+} supported α2 A-domain binding. No binding occurred without addition of divalent cations (data not shown). This supports the ligand-binding assay results that demonstrated a cation-dependent interaction.

α1 A-domain Binds to Collagen IV and Laminin—In addition to collagen I, α1β1 is also reported to bind collagen IV and laminin (16). The binding of collagen IV could not be studied using immobilized A-domains because collagen IV is a poor biotinylation substrate.[2] Antibody detection of α1 A-domain fusion protein binding to immobilized collagen I, collagen IV, and laminin showed that the α1 A-domain bound all these ligands in a concentration-dependent, saturable manner (Fig. 6*A*). The α1 A-domain exhibited very similar levels of binding to collagen I and collagen IV, with laminin bound to a lesser extent. In addition, CB3, an integrin-binding proteolytic fragment of collagen IV, supported a similar level of binding to intact collagen IV although binding was less at lower A-domain concentrations. To test the specificity of the A-domain binding,

[2] D. A. Calderwood, unpublished observation.

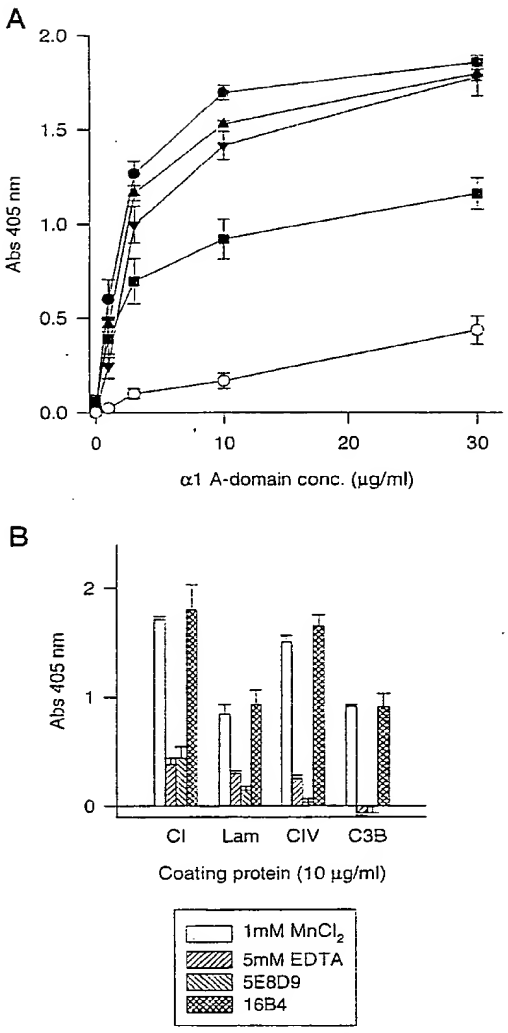

FIG. 6. **α1 A-domain binding to immobilized collagen IV and laminin.** *A*, binding of α1 A-domain fusion protein to collagen I (●), collagen IV (▲), laminin (■), CB3 (▼), and BSA (○) was measured in antibody detection assays. Ligands were coated at 10 μg/ml. Data are mean ± S.E. and $n \geq 6$ from four experiments normalized for 30 μg/ml α1 A-domain binding to collagen I. *B*, A-domain binding assays were used to measure binding of biotinylated fusion protein (0.2 μg/ml) to immobilized ligands coated at 10 μg/ml in the presence of 1 mM $MnCl_2$, 1 mM $MnCl_2$ with 10 μg/ml 5E8D9, 1 mM $MnCl_2$ with 10 μg/ml 16B4, or 5 mM EDTA. Data are mean ± S.E. and $n \geq 9$ from three experiments. Background binding to BSA has been subtracted from each column, and results are normalized for binding to collagen I in the presence of 1 mM $MnCl_2$. *CI*, collagen I; *CIV*, collagen IV; *Lam*, laminin.

we investigated cation dependence and antibody inhibition at low A-domain concentrations. Fig. 6*B* shows that 0.2 μg/ml of biotinylated α1 A-domain bound collagen I, collagen IV, laminin, and CB3 and that binding was inhibited by EDTA and the anti-α1 integrin mAb 5E8D9.

Collagen IV and Laminin Are Ligands for the α2 A-domain—The integrin α2β1 is also a receptor for laminin and collagen IV, so we investigated the binding of α2 A-domain to these ligands and compared it with α1 A-domain. Fig. 7 shows that α2 A-domain bound to collagen IV and laminin; however, in this case, collagen I was a better ligand than collagen IV and laminin. Furthermore, CB3 supported much less binding than collagen IV, unlike the case with α1. Thus, while α1 and α2

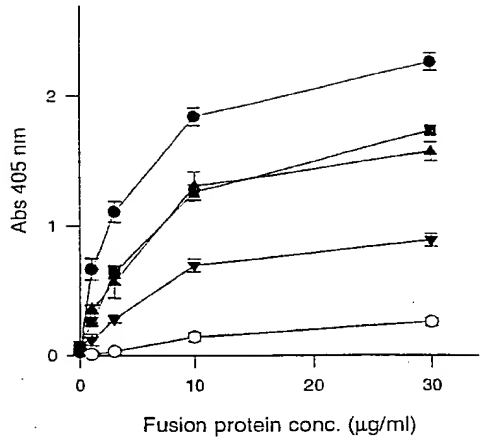

FIG. 7. **A-domain binding to different ligands.** Binding of α2 A-domain fusion protein in 1 mM $MnCl_2$ to collagen I (●), collagen IV (▲), laminin (■), CB3 (▼), and BSA (○) was measured in antibody detection assays. Ligands were coated at 10 μg/ml. Data are mean ± S.E. and $n \geq 6$ from four experiments normalized for 30 μg/ml α2 A-domain binding to collagen I.

A-domains bound the same range of ligands, comparison of Figs. 6*A* and 7 indicate that the relative binding to these ligands differed between the two A-domains. GST showed only background levels of binding to laminin, collagen I, and collagen IV (data not shown).

α1 and α2 A-domain Binding to Collagen IV Fragments—Proteolytic fragments of collagen IV were used to further investigate the differences between α1 and α2 A-domains and the integrin binding sites in collagen IV. As described above, both α1 and α2 A-domains bound to CB3, which is consistent with results obtained for whole integrins in solid phase binding assays and cell attachment assays (12, 18, 19). Further proteolysis of this fragment produces four smaller fragments, F1–F4, and investigation of integrin binding sites in these fragments demonstrated that both α1β1 and α2β1 were able to bind to F1 but only α1β1 bound to F4 (18). F4 lacks the N- and C-terminal regions present in CB3 and F1, which are thought to contain the α2β1 recognition site. Antibody detection of fusion protein binding demonstrated that α1 A-domain bound both F1 and F4 in a concentration-dependent saturable manner, while α2 A-domain bound only poorly, if at all, to F1 and not at all to F4. This binding was inhibited by 5 mM EDTA (data not shown).

Measurement of Apparent Binding Constants for A-domain Collagen Interactions—Binding of α1 and α2 A-domain fusion proteins to collagen I and IV was investigated on a BIAcore. BIAevaluation, the kinetic analysis curve-fitting software supplied with the BIAcore, was used to determine the k_a and k_d values. Prior to fitting the association and dissociation phases of the curve, the binding of A-domain to a blank sensor chip was measured and subtracted from the binding curve. Binding to the uncoated surface was negligible. Binding of a range of fusion protein concentrations (3–100 μg/ml) to collagen I and IV-coated chips was measured. Covalent coupling of collagen I to the chip was much more efficient than for collagen IV, and consequently, signals obtained for binding to collagen I were higher than those to collagen IV. Binding of α2 A-domain to collagen IV could be detected using the BIAcore; however, signals were too low to allow accurate curve fitting. Values obtained are shown in Table I. The BIAevaluation software provides a number of statistical parameters to judge the fitting of the binding model to the experimental data. These indicated that a simple association model provided a good approximation

TABLE I

Kinetic constants for α1 and α2 A-domain interaction with collagens

The k_a and k_d values were generated using the BIAevaluation analysis program, on data recorded for a range of fusion protein concentrations (60–2000 nM) binding to collagen-coated sensor chips. Binding and dissociation was performed in TBS, 1 mM $MnCl_2$. Results are mean ± S.E. ND, not determined; constants for α2 A-domain interaction with collagen IV could not be measured using SPR due to low levels of binding. K_D values were calculated as k_d/k_a from SPR data or from curve fitting analysis of solid phase data, pooled from at least three independent experiments.

A-domain	Ligand	k_a ($\times 10^3$ M^{-1} s^{-1})	k_{dis} ($\times 10^{-5}$ s^{-1})	K_D (nM) (SPR)	n (SPR)	K_D (nM), solid phase
α1	Collagen I	4.1 ± 0.39	8.2 ± 0.61	24 ± 3.4	18	23 ± 2.3
α1	Collagen IV	12 ± 1.3	7.3 ± 2.0	6.0 ± 1.2	9	34 ± 10
α2	Collagen I	67 ± 0.85	120 ± 13	180 ± 25	6	54 ± 6
α2	Collagen IV	ND	ND	ND	ND	115 ± 2

to the experimental data; however, residual plots, comparing the fitted data with experimental results, indicated that a more complex interaction may occur. That the kinetic constants produced provide a good measure of the molecular interactions involved was shown by comparison of a simulated binding curve, produced using the calculated constants, with the experimental results (Fig. 8). This demonstrated that both curves were very similar. The binding curves obtained for A-domain binding to immobilized collagens in solid phase assays can also be fitted to produce apparent K_D values for the interaction (26, 27). These values are shown in Table I and exhibit generally good agreement with those obtained using the BIAcore.

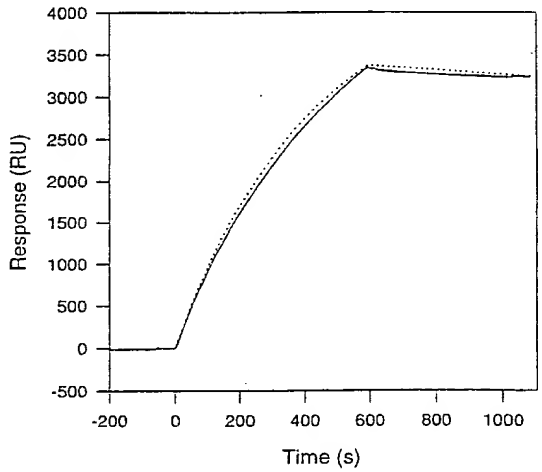

FIG. 8. **Comparison of experimental results for α1 A-domain fusion protein binding to collagen I with a simulated results curve generated using calculated k_a and k_d values.** The experimental sensorgram (*solid line*) was recorded using a flow rate of 5 μl/ml and a 10-min injection of 0.6 μM α1 A-domain. The simulated sensorgram (*dotted line*) was modelled using the experimental parameters and a k_a value of 4×10^3 M^{-1} s^{-1} and k_d value of 8×10^{-5} s^{-1}. To allow comparison of the curves, the signal prior to injection was adjusted to zero, and injection was considered to start at time 0.

DISCUSSION

We have produced a recombinant A-domain from the α1 integrin and compared its ligand binding characteristics with recombinant A-domains from the α2 and αM integrins. Our key findings are that (i) the α1 integrin A-domain is a largely cation-dependent ligand binding domain, (ii) the α1 and α2 A-domains show similar but distinct ligand binding specificities, and (iii) measurement of binding affinities for A-domain-collagen interactions produces values comparable with those reported for integrin-collagen interactions, suggesting that A-domains are the major collagen binding sites in integrins.

Recombinant integrin A-domains and solid phase ligand-binding assays have been used to investigate A-domain-ligand interactions. The results showed that recombinant A-domains from α1 and α2 integrins bind collagen I, collagen IV, laminin, and the collagen IV fragment CB3, but not the αMβ2 ligand fibrinogen. This is the first direct demonstration that the α1 A-domain is a ligand-binding module, and the first observation of laminin binding by A-domains. Interestingly, while the α1 and α2 A-domains were found qualitatively to bind the same ligands, their binding profiles were quantitatively different, and their binding sites within collagen IV appeared to be separate. The specificity of these interactions was confirmed by demonstrating the requirement for native structure of both ligand and A-domain and through antibody inhibition studies.

Published reports of the ligand specificity of α1β1 and α2β1 match these findings with isolated A-domains. Thus, cell binding, antibody blocking, affinity chromatography, and solid phase assays have variously been employed to demonstrate α1β1 binding to collagen types I, III, IV, and VI and to laminin (5, 17, 18, 28–31). In addition, the CB3 fragment of collagen IV and fragments F1 and F4 of CB3 have been shown to contain the α1β1 binding site (18, 19). The α2β1 integrin binds a similar range of ligands (16); however, the affinity of interaction with collagens is different from that of integrin α1β1, and α1β1 appears to bind laminin better than α2β1 (18). The binding site for α2β1 in collagen IV has also been localized to the CB3 fragment (19); however, the α1β1 and α2β1 binding sites are apparently separate (18). The A-domains thus mimic almost all of the ligand binding activity of the intact α1β1 and α2β1 integrins and appear to be key ligand-recognition modules within the intact heterodimer.

To characterize the A-domain-collagen interactions further and address the relative contribution of the A-domain to integrin-ligand binding affinity, the kinetics of binding were measured using SPR. These results showed that the α1 A-domain has a higher affinity for collagens than does the α2 A-domain. Comparison of apparent binding affinities produced from analysis of solid phase binding data indicated a similar pattern of binding; however, the differences between α1 and α2 binding were less notable. The apparent binding constants for α1β1 binding to collagen IV have been measured using both BIAcore and solid phase binding and inhibition assays.[3] These techniques produced K_D values in the range of 1–5 nM, which is in good agreement with the figures obtained for α1 A-domain binding to collagen IV (6–34 nM; Table I). Kern *et al.* (18) have obtained similar values for α1β1 binding to CB3; however, this varied from 1 to 30 nM depending on the divalent cations present. α2β1 binding to CB3 showed similar affinity but was more sensitive to cations (1–110 nM) (18). We measured the affinity of α2 A-domain for collagen IV as 115 nM. Taken together, these findings indicate that the binding affinity of α1 A-domain and α1β1 binding to collagen IV are similar, suggesting that the A-domain is the major ligand binding site in the integrin. The variation in affinity reported for the intact receptor is dependent on divalent cation and suggests that the bind-

[3] J. Eble, unpublished data.

ing site can be regulated by different cations. As discussed below, this may suggest allosteric regulation of the A-domain by cation-binding regions lying outside the A-domain. As the recombinant A-domain is free of this regulation, it may account for the variation between K_D values measured in the presence of Mn^{2+} for the A-domains and those reported for whole integrins. With this in mind, it should be noted that while the simple association model closely approximates the BIAcore data, some non-random variation from the model suggests that a more complex interaction may occur; this may be due to conformational changes in the A-domain.

Integrins are known to require divalent cations for ligand binding, and a number of regions have been proposed to act as cation-binding sites. The crystal structures of the αM and αL A-domains have now been solved and show a single divalent cation-binding site at one end of the domain (4, 32). While it is accepted that intact integrins require cations for ligand binding, cation dependence, independence, and partial dependence have all been reported for isolated A-domain binding to ligands (11, 12, 33, 34). Results reported here show largely cation-dependent binding; however, some variation in cation-dependence was observed. Binding of soluble A-domain to immobilized collagen is only partially cation-dependent, while binding of biotinylated collagen to immobilized A-domain is completely cation-dependent. BIAcore analysis of A-domain-collagen binding showed an absolute requirement for divalent cations, and binding that had already taken place could be reversed with EDTA, suggesting that there is a requirement for cation to remain bound during the integrin-ligand interaction. This suggests that divalent cations are normally required for A-domain ligand binding; however, some cation-independent binding may be seen due to non-native conformations of the recombinant A-domains.

The exact role of divalent cations in ligand binding remains unclear as it is difficult to determine whether the cation binding produces a ligand binding conformation in the A-domain or is itself required as a bridge between ligand and A-domains. Data from mutagenesis and peptide binding studies (14, 11) suggest that cation is not absolutely required for A-domain-ligand binding but that it normally regulates integrin-ligand binding. Qu and Leahy (35) have recently shown that the crystal structures of the recombinant αL A-domain in the presence and absence of divalent cation are very similar, suggesting that the cation-dependence of ligand binding is not due to stabilization of a ligand binding conformation. The presence or absence of divalent cation does, however, have a profound effect on the charge distribution on the cation binding face of the A-domain. As epitope mapping and mutagenesis studies suggest that the ligand binding and cation binding sites are located on the same face of the molecule, surface charge might account for the cation requirement of ligand binding. Our finding that cation was required to maintain ligand binding is consistent with both this and a cation bridge model. In some recombinant A-domains, non-native structures may mean that even in the absence of divalent cation the conformation and charge distribution are such that ligand binding can occur.

In the intact integrin, the situation is further complicated by the presence of other cation-binding sites in the α and β subunits. Cation regulation of the whole integrin does not always match that seen in the isolated A-domain. For example, it is reported that Ca^{2+} will not support collagen binding by α2β1, or α1β1; indeed, Ca^{2+} may actually inhibit Mg^{2+}-induced collagen binding (18, 17). We have shown that, while Ca^{2+} will support only low levels of collagen binding to α2 A-domain, it will allow binding to α1 A-domain. It is possible that, while the A-domain in the intact α1β1 will bind Ca^{2+}, potentially permitting ligand binding, Ca^{2+} binding at other sites on the integrin normally precludes binding.

In conclusion, we have demonstrated that the A-domain from α1 integrin can bind ligands and that laminin is a ligand for A-domains. We demonstrate that distinctions between α1β1 and α2β1 binding to collagen IV are also observed with isolated A-domains and that the affinity of A-domain binding to collagen is similar to that reported for whole integrin binding. Finally, we show that A-domain-ligand binding is largely divalent cation-dependent and suggest that cation, ligand, and A-domain form a ternary complex.

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Contributions of the I and EF Hand Domains to the Divalent Cation-dependent Collagen Binding Activity of the $\alpha_2\beta_1$ Integrin*

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The $\alpha_2\beta_1$ integrin binds collagen in a Mg^{2+}-dependent manner that is inhibited by Ca^{2+}. Like the intact integrin, purified recombinant proteins containing the α_2 integrin I domain, either alone or with variable numbers of α_2 integrin EF hand metal binding sites, bound collagen in a Mg^{2+}-dependent manner, and Ca^{2+} did not support binding. However, unlike the intact integrin, Ca^{2+} did not inhibit the Mg^{2+}-dependent binding of any of the fusion proteins to collagen. Binding to collagen was saturable and blocked by the $\alpha_2\beta_1$ function blocking antibody 6F1. Deletional analysis demonstrated that residues present within the amino-terminal 35 amino acids contribute to the 6F1 epitope and are required for Mg^{2+}-dependent collagen binding. The results indicate that the I domain contains a Mg^{2+} binding site that is essential for collagen binding and that the I domain alone is sufficient for collagen binding. Binding is markedly enhanced in a divalent cation-dependent manner by the addition of the first EF hand motif. Mutation of the EF hand to an inactive form completely abrogated the effect. The sites necessary for Ca^{2+} inhibition are not present within the I domain or the adjacent region containing the three EF hand sites.

The integrins are heterodimeric cell adhesion molecules that mediate cell-cell adhesion and adhesion between cells and the extracellular matrix. They are widely expressed and function throughout development and adulthood in a variety of normal and pathologic processes (for review, see Ref. 1). The $\alpha_2\beta_1$ integrin is expressed on several different cell types, including endothelial and epithelial cells, fibroblasts, lymphocytes, and platelets (2). The ligand specificity of $\alpha_2\beta_1$ varies with cell type. While it serves as a collagen receptor on platelets and fibroblasts, it can serve as both a collagen and as a laminin receptor on endothelial and epithelial cells (3, 4).

Cell adhesion to collagen mediated by the $\alpha_2\beta_1$ integrin is dependent upon the presence of divalent cations (5). Mg^{2+}, for example, supported the adhesion. Ca^{2+} could not substitute for Mg^{2+} and inhibited the Mg^{2+}-dependent adhesion. The adhesion of liposomes containing purified $\alpha_2\beta_1$ integrin to collagen was also found to depend on the presence of Mg^{2+} and to be inhibited by Ca^{2+} (6). The inhibition of Mg^{2+}-dependent adhesion to collagen of liposomes containing the $\alpha_2\beta_1$ integrin occurred via a simple linear noncompetitive mechanism suggesting that Mg^{2+} and Ca^{2+} exert their effects by binding to distinct sites on the $\alpha_2\beta_1$ integrin. Further evidence that Mg^{2+} and Ca^{2+} bind to distinct sites was obtained when limited proteolytic digestion of $\alpha_2\beta_1$ gave different cleavage patterns depending on which divalent cation was present (7).

Several potential divalent cation binding sites present in the α_2 integrin subunit may mediate the distinct effects of Ca^{2+} and Mg^{2+}. Within the extracellular domain of α_2 are three EF hand motifs. These structures were originally described as Ca^{2+} binding sites in regulatory proteins (8, 9) but have since been shown to be capable of binding other divalent cations. The α_2 subunit is a member of a subset of integrin α subunits that contain an approximately 200 amino acid domain located near the amino terminus often referred to as the I (or inserted) domain. Many I domains, including the α_M integrin subunit I domain, contain an additional recently described cation binding site, the metal ion-dependent adhesion site (MIDAS)[1] motif (10). The α_2 I domain also appears to contain a MIDAS motif since all five of the amino acids that contribute to divalent cation coordination in the α_M MIDAS motif are conserved in the α_2 integrin I domain.

I domains share homology with the collagen-binding A domains of von Willebrand factor and cartilage matrix proteins suggesting that integrin I domains may be important determinants in ligand binding. The α_1 subunit has been shown to be involved with the binding of $\alpha_1\beta_1$ to its ligands, collagen, and laminin (11). Likewise, the α_M I domain is required for the interaction of the $\alpha_M\beta_2$ integrin with its ligands, ICAM-1, iC3b, and fibrinogen (10, 12). The α_L I domain also appears to be important in the binding of the integrin $\alpha_L\beta_2$ with its ligands, ICAM-1, and ICAM-3 (13, 14). Similarly, several lines of evidence implicate the involvement of the α_2 I domain in ligand binding activity of the $\alpha_2\beta_1$ integrin. First, a polyclonal antiserum directed against a bacterially expressed α_2 I domain fusion protein was shown to block the attachment of endothelial cells to gelatin, type I collagen, and laminin (15). Second, a series of human/bovine α_2 integrin chimeras was generated and used to map the epitopes recognized by anti-human α_2 integrin monoclonal antibodies that were capable of inhibiting the ligand binding activity of the human $\alpha_2\beta_1$ integrin. All of these antibodies mapped to regions within the α_2 I domain, revealing the significance of the I domain with regard to collagen recognition (16). Finally, several mutagenesis studies have demonstrated the importance of amino acids within the α_2 integrin I domain for ligand binding (16, 17). Recently, recombinant α_2 integrin I domain expressed in bacteria as a glutathione *S*-transferase (GST) fusion protein has been shown to bind specifically to collagen. However, two reports (17, 18) have presented conflicting data as to whether the I domain, like the intact integrin, binds collagen in a divalent cation-dependent manner.

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[1] The abbreviations used are: MIDAS, metal ion-dependent adhesion site; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay.

To elucidate the roles of the various divalent cation binding sites present within the α_2 subunit with regard to the metal ion-dependent function of the intact $\alpha_2\beta_1$ integrin, we have expressed a series of α_2 integrin I domain-containing proteins with either none, one, two, or all three of the EF hand sites. Our results indicate an essential role for the I domain and a heretofore unrecognized role for the first EF hand motif in divalent cation-dependent collagen binding activity.

EXPERIMENTAL PROCEDURES

Cloning and Expression of Integrin α_2 I Domain-containing Proteins—Complementary DNAs encoding the human α_2 integrin subunit I domain and the I domain with one, two, or all three of the EF hand divalent cation binding sites were generated by PCR using full-length human α_2 integrin cDNA as template. The proteins encoded by this series constructs will be referred to as I, I + 1, I + 12, and I + 123. All four of the proteins in this series begin at Ser-124 and terminate at Met-349, Gly-516, Lys-570, and Ser-620 of the published α_2 sequence (19). In addition, cDNAs encoding a shorter I domain protein lacking the 35 amino-terminal amino acids and the analogous I + 1 protein were also prepared. These shorter proteins, referred to as ΔI and ΔI + 1, begin at Trp-159 and terminate at Met-349 and Gly-516, respectively. Thus these two proteins lack the DXSXS portion of the MIDAS motif. The PCR primers were designed such that all of the amplification products would contain a *Bgl*II restriction site at their 5′ ends and a stop codon followed by an *Xho*I restriction site at their 3′ ends. The PCR products were digested with *Bgl*II and *Xho*I, purified in agarose gels, and cloned into *Bam*HI and *Xho*I-digested GST fusion protein expression vector pGEX-5X-1 (Pharmacia Biotech Inc.). The sequences of all cDNAs used in this study were determined using the dideoxy chain termination method (20) and compared with the published α_2 integrin sequence (19).

The sequences of the oligonucleotides used for PCR were as follows: I domain forward primer, 5′-GAAGATCTCTCCTGATTTTCAGCTCTCAGCCAGC-3′; I domain reverse primer, 5′-CCGCTCGAGTCACATTTCCATCTGAAAGTTGTCTCC-3′; I + 1 reverse primer, 5′-CCGCTCGAGTCAGCCTTCAAGAAATTGGTGCTGACC-3′; I + 12 reverse primer, 5′-CCGCTCGAGTCACTTTGTGCGGATAGTGCCCTGATG-3′; I + 123 reverse primer, 5′-CCGCTCGAGTCATGACCAGAGTTGAACCACTTGTCC-3′; ΔI forward primer, 5′-GAAGATCTGGGATGCAGTAAAGAATTTTTTGG.

To prepare an I + 1 protein with a mutated EF hand, pGEX-5X-1/I + 1 was digested with *Pst*I and *Xho*I, and the 357-base pair *Pst*I-*Xho*I fragment was purified in an agarose gel. The fragment was cloned into pBlueScript KS, previously digested with the same enzymes. The Kunkel (21) method of site directed mutagenesis was used to create a double mutant, D272KD→AKA. This protein will be referred to as I + 1*. The oligonucleotide used for the mutagenesis reaction was antisense; its sequence was 5′-CACGTCTGTAATGGTGGCTTTAGCCACATCAACTGAAC-3′. The mutation was verified by sequencing, and the 357-base pair *Pst*I-*Xho*I fragment containing the double mutation was cloned back into the pGEX-5X-1/I + 1 background. Fig. 1 shows a schematic diagram of the constructs used in this study.

Trial inductions were performed to determine whether the selected clones could direct expression of appropriately sized GST fusion proteins. *E. coli* DH5α containing each of the plasmid constructs was grown at 37 °C in 60 ml of 2 × YT media supplemented with 0.2% glucose and 100 μg/ml ampicillin. Uninduced samples were removed from each culture when the A_{550} reached 0.3–0.4. Isopropylthiogalactoside was then added to a final concentration of 1 mM, and the cultures were returned to the incubator for 3 h to allow for accumulation of the expressed proteins. Cell lysates from the uninduced and induced samples were analyzed by SDS-PAGE (22) followed by Coomassie Blue staining. All of the constructs directed the expression of recombinant proteins of the expected size. The site of accumulation and degree of solubility was determined for a representative I domain-containing protein using a published cellular fractionation protocol (23). Bacteria harboring the I + 123 construct were grown and induced as described above. At the end of the induction period, the sample was fractionated into media, periplasmic, cytoplasmic soluble, membrane, and insoluble fractions. Each fraction was analyzed by SDS-PAGE, followed by Coomassie Blue staining. The bulk of the recombinant protein (95–100%) accumulated in the insoluble fraction. The other I domain-containing proteins accumulated in the insoluble fraction as well.

For the purification of the fusion proteins, the inductions were performed as above except that the culture volume was increased to 500 ml. At the end of the induction period, the cells were recovered by centrifugation at 2600 × *g* for 10 min. The cells were washed twice with 10 ml of ice-cold phosphate-buffered saline (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.4) and stored at −70 °C until needed. Insoluble fractions were prepared and washed using the Triton X-100 procedure described by Marston (24). The recombinant proteins were solubilized in 8 M urea on ice for 1 h. After removal of urea-insoluble material by centrifugation at 12,100 × *g* for 20 min, the urea was diluted to 1.33 M with 25 mM Tris-HCl, pH 8.0, 10 mM EDTA. The proteins were purified by affinity chromatography on glutathione-Sepharose (Pharmacia) according to a published method (25). Following purification, the proteins were dialyzed extensively against TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). Protein yields were determined using the BCA protein assay reagent (Pierce). Fig. 2 shows a Coomassie Blue stained SDS-PAGE gel containing approximately 5 μg/lane of each of the recombinant proteins used in this study. All recombinant proteins were subjected to gel filtration analysis on a 10 × 300 mm Superose 12 column (Pharmacia) equilibrated with TBS containing 2 mM $MgCl_2$ (Fig. 3).

Collagen and Laminin Binding Assays—The wells of a 96-well microtiter plate (Immulon 2, Dynatech Laboratories, Inc.) were coated overnight at 4 °C with 0.1 ml of 30 μg/ml collagen I from calf skin (Sigma) in 0.09% acetic acid or with 30 μg/ml laminin I (Collaborative Biomedical Products) in TBS. The wells were washed twice with 0.15 ml TBS and then blocked for 1 h at room temperature with 0.15 ml of 100 μg/ml bovine serum albumin (ICN Biomedicals, Inc.) in TBS. Recombinant proteins were diluted to 400 nM in various wash buffers (TBS containing 0.05% Tween-20, 10 μg/ml bovine serum albumin, and either 1 mM EDTA, 2 mM $CaCl_2$, 2 mM $CaCl_2$ plus 2 mM $MgCl_2$, or 2 mM $MgCl_2$). The wells were washed once with 0.15 ml of the appropriate wash buffer, and then 0.1 ml of each recombinant protein was added and allowed to interact for 1.5 h at room temperature. Wells were then washed three times with 0.15 ml of the appropriate wash buffer, and then 0.1 ml of a 1:500 dilution of anti-GST antiserum (Pharmacia) in the appropriate wash buffer was added for 1 h at room temperature. Following this incubation, the wells were again washed three times, and then 0.1 ml of a 1:4500 dilution of pig-anti-goat secondary antibody-horseradish peroxidase conjugate (Boehringer Mannheim) in the appropriate wash buffer was added for 1 h at room temperature. The wells were again washed three times, and 0.1 ml of tetramethylbenzidine dihydrochloride (Sigma) prepared according to the manufacturer directions was added per well. After 1 h of substrate conversion, reactions were stopped with 0.025 ml of 4 N H_2SO_4, and the plates were read at 450 nm.

ELISA—I domain-containing proteins were diluted to 10 μg/ml in TBS containing 2 mM $MgCl_2$ and used to coat the wells of a 96-well microtiter plate (Immulon 2, Dynatech). Coating was carried out overnight at 4 °C with 0.1 ml of solution/well. The wells were washed twice with 0.15 ml of TBS containing 2 mM $MgCl_2$ and then blocked for 1 h at room temperature with 0.15 ml of TBS containing 100 μg/ml bovine serum albumin and 2 mM $MgCl_2$. Primary antibodies used include anti-GST antiserum and anti-human α_2 monoclonal antibodies 6F1 and 12F1. The anti-GST antiserum was diluted 1:2500; the monoclonal antibodies were diluted to 1 μg/ml in wash buffer (TBS containing 0.05% Tween-20, 10 μg/ml bovine serum albumin, and 2 mM $MgCl_2$). Following blocking, the wells were washed once with 0.15 ml of wash buffer, and then 0.1 ml of primary antibody was added and allowed to interact for 1 h at room temperature. The wells were washed three times with 0.15 ml of wash buffer, and then 0.1 ml of secondary antibody-horseradish peroxidase conjugate (pig-anti-goat for anti-GST or goat-anti-mouse for 6F1 and 12F1) diluted 1:4500 in wash buffer was added per well. Substrate was added, and the plates read as described above.

Antibody Blocking Assay—The wells of a 96-well microtiter plate were coated with collagen and blocked with bovine serum albumin as described above. I + 1 (100 nM) was preincubated with anti-human α_2 antibodies 6F1 or 12F1 (300 nM) for 1 h at room temperature in wash buffer (TBS containing 0.05% Tween-20, 10 μg/ml bovine serum albumin, and 2 mM $MgCl_2$). The wells were washed once with 0.15 ml of wash buffer, and then 0.1 ml of I + 1/antibody mixture was added and allowed to interact for 1.5 h at room temperature. Detection of collagen bound I + 1 was carried out as described above. Substrate was added, and the plates were read at 450 nm.

RESULTS

As an approach to assess the contributions of distinct classes of divalent cation binding sites present within the α_2 integrin

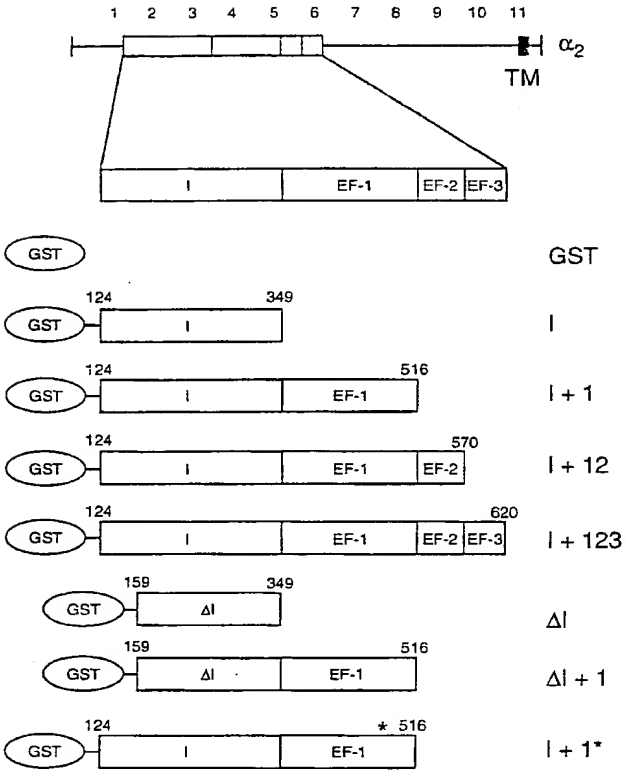

FIG. 1. **Schematic representation of recombinant α_2 integrin I domain-containing GST fusion proteins.** *Numbers* above the full-length α_2 subunit (*top line*) refer to amino acid residue position/100. *Numbers* above each construct refer to amino acid position in the α_2 integrin subunit. The *asterisk* in I + 1* denotes the double mutation D272KD→AKA in the EF hand motif.

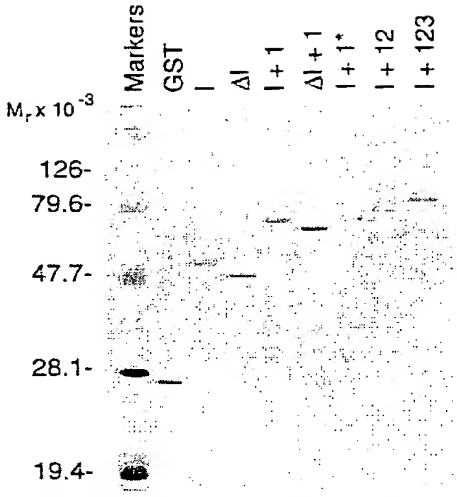

FIG. 2. **SDS-PAGE gel containing purified I domain-containing GST fusion proteins.** Proteins were stained with Coomassie Blue. 5 μg of protein were loaded per lane.

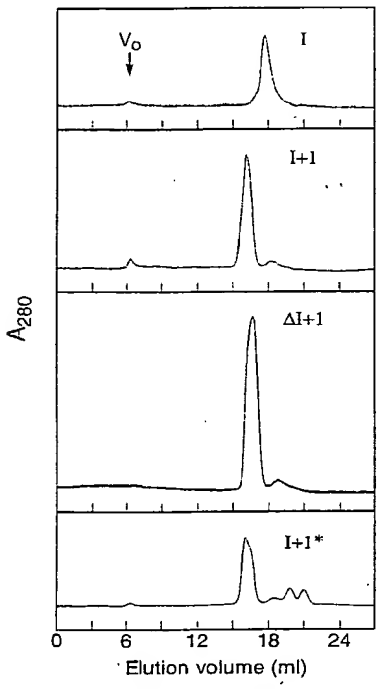

FIG. 3. **Gel filtration analysis of recombinant α_2 integrin I domain-containing proteins.** 20–40 μg quantities of the indicated proteins were analyzed on a Superose 12 column (Pharmacia) equilibrated with TBS containing 2 mM $MgCl_2$. The position of the void peak is indicated.

subunit to the collagen binding activity of the $\alpha_2\beta_1$ integrin, the domains were expressed as recombinant GST fusion proteins containing the α_2 integrin I domain alone or in combination with one, two, or all three of the EF hand-like motifs (I, I + 1, I + 12, I + 123). In addition, the I domain was modified by deleting 35 amino acids from its amino terminus in two constructs (ΔI and ΔI + 1). The EF hand motif of I + 1 was modified by incorporating two D→A point mutations to render the EF hand motif incapable of metal binding (I + 1*). The recombinant proteins examined in this investigation are presented schematically in Fig. 1.

After purification by affinity chromatography on glutathione-Sepharose, the proteins were subjected to analysis by SDS-PAGE (Fig. 2). The recombinant proteins were further analyzed by gel filtration chromatography. This analysis confirmed the purity of the proteins in agreement with the SDS-PAGE analysis and revealed the lack of protein aggregation with less than 2.6% of the protein running with an apparent size larger than that predicted for the monomeric species. Quantitative analysis of I domain and I + 1 proteins revealed that 95 and 96%, respectively, of the protein applied to the column eluted in the monomer peaks. Representative profiles for the I, I + 1, ΔI + 1 and I + 1* proteins are shown in Fig. 3. Similar chromatographic profiles were obtained for each of the other proteins used in this study (data not shown).

The proteins were then tested for collagen binding activity. To assess the divalent cation specificity, if any, collagen binding assays were conducted in the presence of 2 mM EDTA, 2 mM Ca^{2+} or 2 mM Mg^{2+}. The ability of Ca^{2+} to inhibit any Mg^{2+}-dependent collagen binding was assessed by carrying out the binding assay in the presence of 2 mM of both Ca^{2+} and Mg^{2+}. The results are shown in Fig. 4. GST alone did not bind specifically to collagen under any of the divalent cation conditions. As previously observed with the intact $\alpha_2\beta_1$ integrin, all four of the proteins containing an intact I domain bound collagen in a Mg^{2+}-dependent manner. As also observed with the intact integrin, Ca^{2+} did not support the collagen binding activity of any of the I domain-containing proteins. However, unlike the intact $\alpha_2\beta_1$ integrin, Mg^{2+}-dependent collagen binding activity of the I domain-containing proteins was not inhibited by Ca^{2+}.

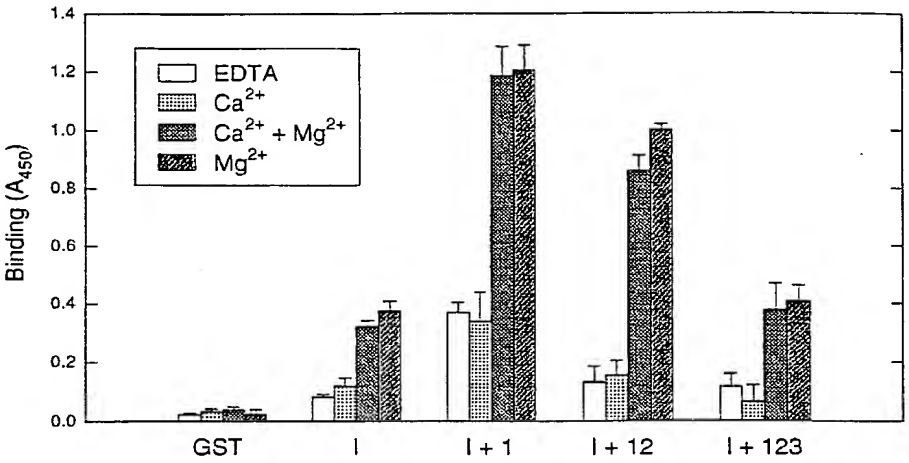

FIG. 4. **Binding of GST and I domain constructs to collagen.** Collagen binding was measured in the presence of 1 mM EDTA, 2 mM Ca^{2+}, 2 mM each of Ca^{2+} and Mg^{2+}, or 2 mM Mg^{2+}.

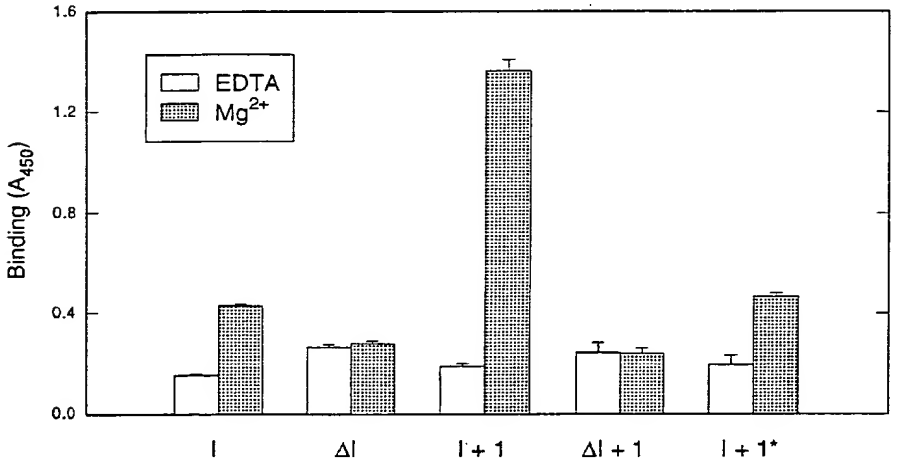

FIG. 5. **Binding of I and I + 1 proteins to collagen.** I, ΔI, I + 1, ΔI + 1, and I + 1* were tested for binding to collagen in the presence of 1 mM EDTA or 2 mM Mg^{2+}.

Addition of the first EF hand motif onto the I domain appeared to markedly enhance Mg^{2+}-dependent collagen binding activity as revealed by an increased extent of binding. This was most apparent in the I + 1 and I + 12 proteins.

The necessity of an intact I domain for the collagen binding activity of the constructs was examined with two fusion proteins containing truncated I domains in which the amino-terminal 35 residues were deleted (ΔI and ΔI + 1). The deleted region contained the DXSXS sequence, a region thought to be critical for the structural integrity of the MIDAS motif (10). As expected and as shown in Fig. 5, both the I and I + 1 constructs bound collagen in a Mg^{2+}-dependent manner. However, neither of the truncated constructs (ΔI and ΔI + 1) bound collagen. Thus an intact MIDAS motif is required for Mg^{2+}-dependent collagen binding activity.

The contribution of the putative metal binding sequences present within the first EF hand motif to the enhanced collagen binding activity of the I + 1 protein relative to the I domain was examined by mutating two aspartate residues essential for metal binding activity of the motif (Asp-272 and Asp-274) to alanines to create the I + 1* protein. Unlike the wild-type EF hand motif, the mutated EF hand conferred no enhanced collagen binding activity upon the I domain (Fig. 5). The collagen binding activity of I + 1* was comparable with that of I domain alone. Thus, sequences within the EF hand motif that confer metal binding properties upon the motif, are essential for the enhancement of collagen binding activity.

The enhanced collagen binding activity of the I + 1 construct relative to that of the I domain alone was examined in greater detail and over a range of concentrations (Fig. 6). Both the I and I + 1 proteins bound to collagen in a concentration-dependent and saturable manner. Whereas half-maximal binding of the I domain protein to collagen occurred at 820 nM, the half-maximal binding of the I + 1 construct was observed at 87 nM.

Mg^{2+} concentrations required for half-maximal collagen binding were determined for I domain, I + 1, I + 12, and I + 123 proteins and found to be 0.54, 0.31, 0.61, and 1.17 mM, respectively (Fig. 7). Mn^{2+} was also shown to support collagen binding of each of these constructs (Fig. 8). Approximately 0.5 mM Mn^{2+} was required for half-maximal collagen binding.

Anti-human α_2 integrin monoclonal antibodies 6F1 and 12F1 were tested for their ability to bind the I, ΔI, I + 1, and ΔI + 1 fusion proteins. The results are shown in Fig. 9A. All of the proteins were recognized by the anti-GST antiserum to similar extents, indicating that comparable quantities of the proteins were coated onto the microtiter wells. Monoclonal antibodies 6F1 and 12F1 both bound equivalently to the I and I + 1 proteins that contained intact I domains. Neither bound to the ΔI and ΔI + 1 proteins containing truncated I domains. Both of the antibodies also effectively recognized the I + 1*, I + 12, and I + 123 proteins (data not shown). The same patterns of reactivity were observed in the presence of either 2 mM Mg^{2+} or 2 mM EDTA (data not shown). Thus, the two distinct complex, conformation-dependent epitopes recognized by the 6F1 and

12F1 antibodies (26, 27) are retained to comparable extents in the I and I + 1 constructs, suggesting that the conformation of the I domain in these two proteins is intact. Antibody 6F1 which inhibits the binding of the intact $\alpha_2\beta_1$ integrin to collagen (26), also effectively blocked the binding of the I + 1 protein to collagen. 12F1, an antibody that does not inhibit the binding of the intact $\alpha_2\beta_1$ integrin to collagen (28), similarly failed to inhibit the binding of I + 1 protein to collagen although, as shown in Fig. 9*A*, the 12F1 antibody effectively bound to the I + 1 protein. The effects of the 6F1 and 12F1 antibodies on collagen binding activity of the I + 1 protein are shown in Fig. 9*B*.

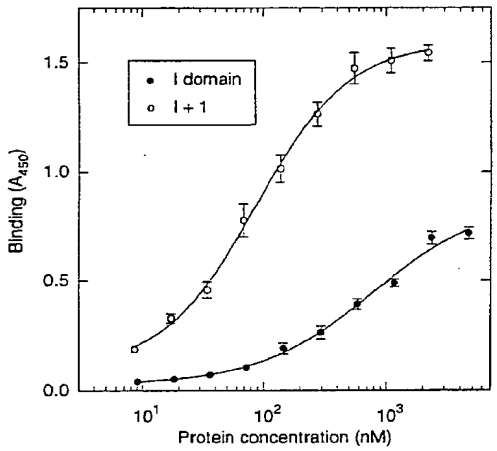

FIG. 6. **Saturable binding of I and I + 1 to collagen.** Various concentrations of I and I + 1 were tested for binding to collagen in the presence of 2 mM Mg^{2+}.

Since the I and I + 1 proteins exhibited rather different collagen binding activities, we next examined the binding of the two proteins to laminin, a second ligand for the $\alpha_2\beta_1$ integrin. When examined in either Mg^{2+} or Mn^{2+}-containing buffers, the I and I + 1 proteins bound laminin to comparable extents (Fig. 10). Unlike binding to collagen, however, binding to laminin was markedly enhanced in the presence of Mn^{2+}. Both I and I + 1 proteins were similarly affected.

DISCUSSION

The adhesion of cells to collagen via the $\alpha_2\beta_1$ integrin requires the presence of Mg^{2+} (5). Ca^{2+} is incapable of supporting $\alpha_2\beta_1$ integrin-mediated adhesion to collagen and inhibits the Mg^{2+}-dependent adhesion. Liposomes containing purified $\alpha_2\beta_1$ integrin demonstrated identical metal ion dependence (6). Recently, the I domains of several integrins have been shown to be important determinants of ligand binding (10–14). Evidence of the involvement of the α_2 I domain in collagen recognition includes: (*a*) an anti-α_2 antiserum blocks endothelial cell attachment to collagen (15); (*b*) several $\alpha_2\beta_1$ integrin function blocking antibodies map to the I domain (16); and (*c*) purified recombinant α_2 I domain binds specifically to collagen (17, 18). The crystal structure of the I domain of the related α_M integrin subunit has been solved and found to contain a single novel Mg^{2+} binding site that involves residues that are widely separated in the primary sequence (10). In addition to the Mg^{2+} binding MIDAS motif within the I domain, the α_2 integrin subunit contains three EF hand-like metal binding sites in close proximity to the I domain. In the present study, we have prepared a series of α_2 I domain-containing GST fusion proteins with various numbers of EF hand sites for the purpose of establishing the contributions of the divalent cation binding

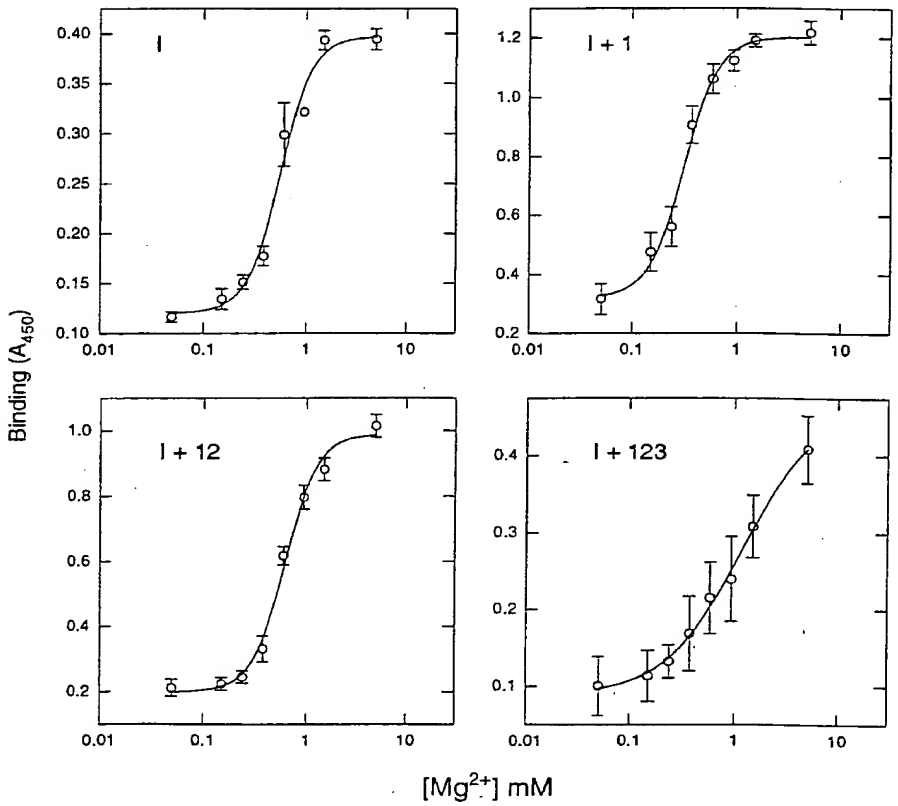

FIG. 7. **Mg^{2+}-dependence of binding of I domain constructs to collagen.** Binding of the I domain-containing GST fusion proteins to collagen in the presence of the indicated concentrations of Mg^{2+} was determined.

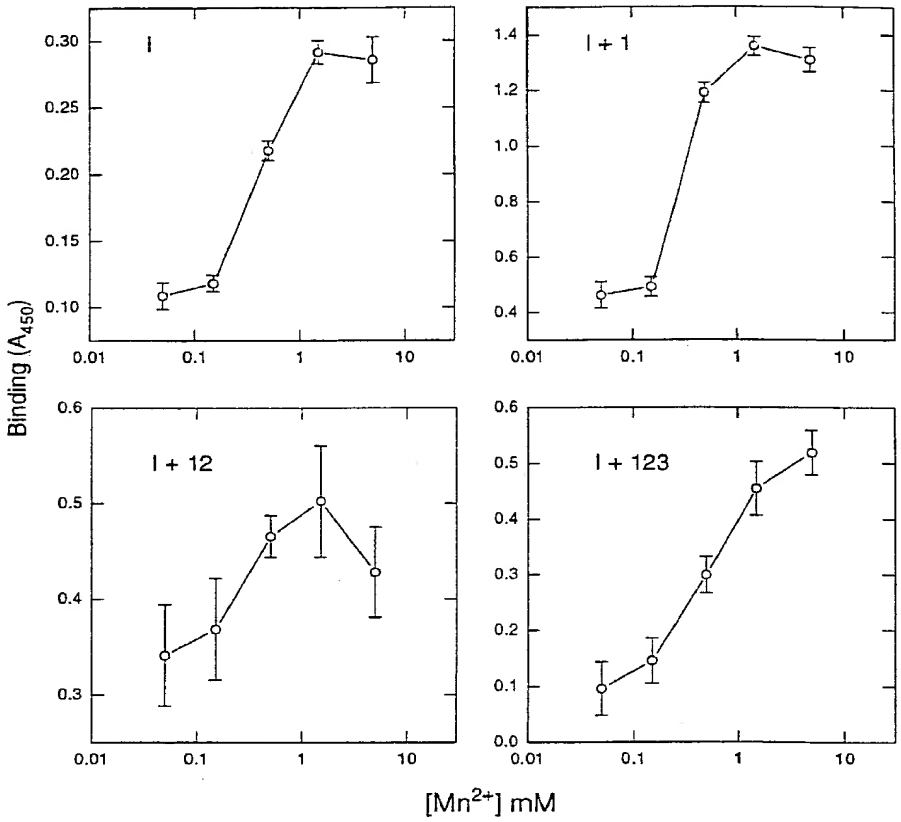

FIG. 8. **Mn^{2+}-dependence of binding of I domain constructs to collagen.** Binding of the I domain-containing GST fusion proteins to collagen in the presence of the indicated concentrations of Mn^{2+} was determined.

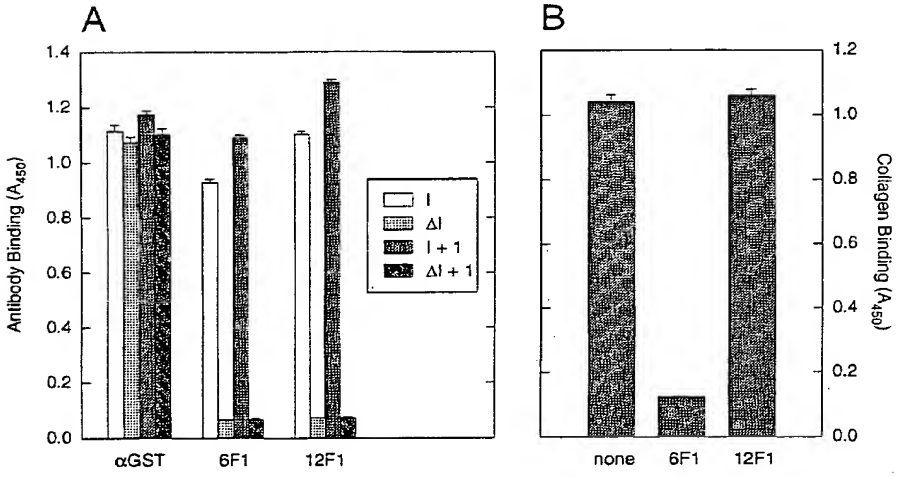

FIG. 9. **Antibody recognition and blocking.** *A*, anti-GST antiserum and anti-human α_2 monoclonal antibodies 6F1 and 12F1 were tested by ELISA for reactivity with I domain and I + 1 proteins. *B*, monoclonal antibodies 6F1 and 12F1 were tested for their ability to inhibit the binding of I + 1 to collagen. I + 1 (100 nM) was preincubated with either antibody (300 nM) for 1 h and then tested in the collagen binding assay.

sites present within the I and EF hand domains of the α_2 integrin subunit to the metal and ligand binding properties of the integrin.

Like the intact integrin, each of the I domain-containing proteins bound collagen in a Mg^{2+}-dependent manner. As also observed with the intact integrin, Ca^{2+} could not substitute for Mg^{2+}. Unlike the intact integrin, however, Ca^{2+} did not inhibit the Mg^{2+}-dependent binding to collagen of any of the I domain-containing proteins. These data suggest that the sites responsible for Ca^{2+} inhibition of Mg^{2+}-dependent collagen binding activity are either not present within the I domain or the region containing the three EF hand structures or that their inhibitory effects are not manifested outside of the context of the intact integrin. The finding that isolated I domain binds collagen in a Mg^{2+}-dependent manner is in agreement with the recent work of Tuckwell, *et al.* (18) but contrasts with results from Kamata and Takada (17), who found that independently expressed I domain bound collagen in a divalent cation-independent manner. The reason for the discrepancy is not apparent but may represent an adverse consequence of the iodination of the recombinant I domain protein. We have previously observed deleterious effects on $\alpha_2\beta_1$ integrin structure and ligand binding activity as a result of iodination (7). The results of our binding studies using the ΔI and ΔI + 1 proteins strongly

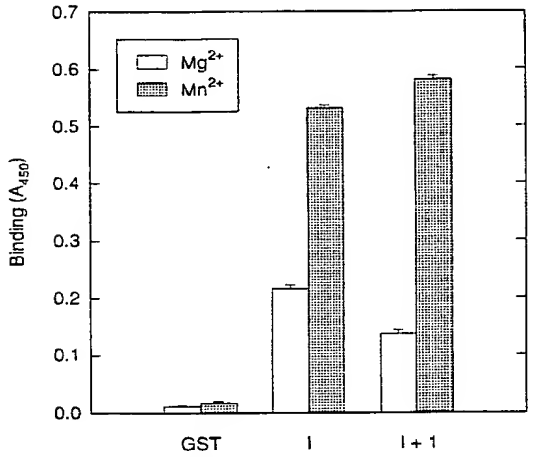

FIG. 10. **Binding of I and I + 1 proteins to laminin.** Binding of GST, I, and I + 1 proteins to laminin was determined in the presence of 1 mM EDTA, 2 mM Mg^{2+}, or 2 mM Mn^{2+}. The results are expressed as divalent cation-dependent binding observed in the presence of Mg^{2+} or Mn^{2+}.

support the conclusion that the MIDAS site present within the I domain is critical for the collagen binding activity of the α_2 I domain.

A marked enhancement of collagen binding activity was consistently observed upon the addition of the first EF hand motif to the I domain. To determine if the increased binding was contingent upon the divalent cation binding activity of the EF hand, an I + 1 protein with a mutated EF hand was prepared. To ensure complete inactivation the EF hand site in I + 1, aspartates at positions 3 and 5 were mutated to alanines. Mutation of the EF hand site to an inactive form completely abrogated the increase in collagen binding observed for I + 1, reducing its collagen binding activity to that of the I domain. This finding indicates that the enhancement was likely a direct result of Mg^{2+} binding to the EF hand rather than to divalent cation-independent interactions of other regions of the added sequence.

Mg^{2+} concentrations required for half-maximal binding to collagen of I domain, I + 1, I + 12, and I + 123 proteins ranged from 0.3 to 1.2 mM. These values are similar to those observed for half-maximal $\alpha_2\beta_1$ integrin-mediated adhesion of platelets and of other cells to collagen (5, 29). Mn^{2+} has been shown to support the adhesion of cells to collagen at significantly lower concentrations than Mg^{2+} (10–30 μM) (29, 30). However, the I domain fusion proteins required approximately 0.5 mM Mn^{2+} for half-maximal collagen binding. This concentration is similar to that of Mg^{2+} rather than the ten-fold lower concentration observed with the intact integrin. These data suggest that for the I domain-containing proteins, Mn^{2+} is substituting for Mg^{2+}.

Recently, the I domain from the α_M integrin subunit has been crystallized in the presence of a limiting concentration of Mn^{2+} (31), and the structure obtained from this analysis was compared with that obtained when the protein was crystallized in the presence of Mg^{2+} (10). A change in conformation as well as a change in the way the metal ion was coordinated was revealed when the protein was crystallized in the presence of Mn^{2+}. The differing divalent cation-dependent structures may potentially reflect affinity modulation of the integrin. Our data indicate that the conformational changes observed in the I domain are themselves insufficient to confer significantly altered collagen binding activity on the I domain protein(s). In contrast, Mn^{2+} did markedly enhance binding of the I and I + 1 proteins to laminin. In this regard, it is noteworthy that only the most activated form of the $\alpha_2\beta_1$ integrin is thought to exhibit laminin binding activity (32).

The antibody binding experiments are significant in their own right. Both the 6F1 and 12F1 monoclonal antibodies bound to the proteins containing an intact α_2 integrin I domain (I, I + 1, etc.) in the absence of the β_1 integrin subunit indicating that the β_1 subunit is not necessary for formation of the epitope recognized by these two antibodies. Deletion of the amino-terminal 35 amino acids of the I domain (residues 124–158 of the published α_2 integrin sequence (19)) to form the ΔI and ΔI + 1 proteins resulted in loss of reactivity with both the 6F1 and 12F1 antibodies. Since the deletion destroyed the integrity of the MIDAS motif, it was conceivable that critical divalent cation-dependent structures essential for reactivity with the antibodies may have also been destroyed. This seemed unlikely, however, since the same patterns of antibody reactivity were observed in both EDTA and Mg^{2+}-containing buffers. These data, therefore, suggest that in addition to the region of residues 173–259 identified by Kamata, *et al.* (16) in their study of human/bovine chimeric α_2 integrin, an additional determinant present within residues 124–158 makes an important contribution to the apparently complex epitopes recognized by the 6F1 and 12F1 antibodies.

In summary, the results of this investigation indicate, in agreement with other recent studies, that the α_2 integrin subunit I domain is sufficient for collagen binding activity. The data obtained in this study indicate that, while there appears to be a Mg^{2+} binding site within the I domain that is critical for collagen binding, the sites responsible for Ca^{2+} inhibition are not present within the I domain or the region containing the three EF hand structures or that additional portions of the intact integrin are also required in conjunction with Ca^{2+} binding to observe the inhibitory effect. These results are consistent with our earlier observation that Mg^{2+} and Ca^{2+} exert their effects by binding to distinct sites on $\alpha_2\beta_1$ (6, 7).

Finally, our studies reveal several differences between the binding of the I and I + 1 proteins to collagen and laminin. Unlike binding to collagen, which was equivalent in the presence of Mg^{2+} or Mn^{2+}, binding of both the I and I + 1 proteins to laminin was greatly enhanced in the presence of Mn^{2+}. Furthermore, whereas binding to collagen was considerably enhanced by the addition of the first EF hand motif to the I domain, binding to laminin was essentially unaltered. The structural basis underlying these apparent mechanistic differences warrants further exploration.

Acknowledgments—We are grateful to Drs. Barry S. Coller and Virgil L. Woods, Jr., for generous gifts of the monoclonal antibodies 6F1 and 12F1, respectively. We thank Nancy L. Mathis for technical assistance.

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Direct Binding of Collagen to the I Domain of Integrin α2β1 (VLA-2, CD49b/CD29) in a Divalent Cation-independent Manner*

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Integrin α2β1 is a major divalent cation-dependent receptor for collagen. Here, we show that the recombinant inserted/interactive domain (I domain) of α2 specifically interacts with collagen, indicating the I domain contains all the components necessary for collagen binding. Evidence was obtained that divalent cations are not required for collagen binding to the I domain fragment, indicating that divalent cations are not involved in the actual binding to collagen but probably in the regulation of the binding. We identified Thr-221 within the previously identified putative ligand binding region as a residue critical for collagen binding to both α2β1 and the I domain fragment. Thr-221 may be involved in the actual collagen binding and recognition.

Integrins are a supergene family of cell adhesion receptors. This family is composed of at least 13 α subunits and 8 β subunits. Integrins recognize multiple ligands and mediate cell-cell and cell-extracellular matrix interactions in a divalent cation (Ca^{2+}, Mg^{2+}, and/or Mn^{2+})-dependent manner (1–6).

Integrin α2β1 is a major collagen receptor in fibroblasts, platelets, and leukocytes (7–12). α2β1 contributes to the three-dimensional type I collagen gel contraction and the reorganization of collagen by fibroblast (13, 14). α2β1 is also involved in the migration of tumor cells within collagenous matrices (6, 15) and in platelet aggregation induced by collagen (16). The α2β1 is also a receptor for echovirus 1 (17, 18). α2β1 has also been shown to be involved in cell-cell interaction (19, 20), probably by interacting with α3β1 (21).

The α2 chain consists of a large extracellular domain, a transmembrane domain, and a short cytoplasmic segment (22). In addition to common characteristics of integrin α chain (the well conserved Cys residues, three metal binding domains of the general structure DXDXDGXXD, and transmembrane domain), α2 chain has an inserted/interactive domain (I domain)[1] of about 200 amino acids located close to the three metal binding sites. I domains are also present in other proteins, including von Willebrand factor (23), cartilage matrix protein (24), collagen type VI (25, 26), complement C2 (27), factor B (28), the αL subunit of lymphocyte function-associated antigen-1 (αLβ2), the αM subunit of Mac-1 (αMβ2) (29–31), the αX subunit of p150,95 (32), the α1 subunit of α1β1 (33, 34), and the αE subunit of αEβ7 (35).

Recently, we have shown that anti-α2 monoclonal antibodies (mAbs) that block collagen and/or echovirus 1 binding recognize the I domain, suggesting that the I domain may be involved in the ligand binding (36). Also, divalent cation binding sites have been found in the I domain of αM. Asp-140 and Asp-242 within the αM I domain are critical for the cation binding, and mutations of the 2 residues block binding of αMβ2 to iC3b (37). The corresponding residues in the α2 I domain (Asp-151 and Asp-254) were shown to be critical for collagen binding of α2β1 integrin as well (36).

In the present study, we examined the ligand/α2β1 interaction using a fusion protein that spanned the α2 I domain. We report here that the recombinant I domain specifically binds to collagen, but the binding is divalent cation-independent. These data suggest that the I domain has all the necessary components for collagen binding and that divalent cations are not involved in the actual binding of collagen to the I domain. Consistent with these data, mutations of Asp-151 and Asp-254, which are critical for divalent cation binding to the I domain, do not affect or only partially inhibit the collagen binding to the I domain fragment. By contrast, Thr-221 is critical for collagen binding to both α2β1 and the I domain fragment.

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies—5E8 (38) was obtained from R. B. Bankert (Roswell Park Cancer Institute, Buffalo, NY), 6F1 (39) from B. Collar (State University of New York at Stony Brook, NY), 12F1 (8, 40) from V. Woods (University of California San Diego, CA), AA10 (17) from J. Bergelson and R. W. Finberg (Dana-Farber Cancer Institute, Boston, MA), P1H5 and P4B4 from W. G. Carter (F. Hutchinson Cancer Institute, Seattle, WA) and E. Wayner (University of Minnesota, Minneapolis, MN), P1E6 from Telios Pharmaceuticals (San Diego, CA), RMAC11 (41) from R. Faull (St George Hospital, Kogarah, Australia), and HAS3 and HAS4 (42) from F. Watt (Imperial Cancer Research Fund, London, United Kingdom).

Preparation of Glutathione S-Transferase-I Domain Fusion Protein (GST-α2I)—A cDNA fragment encoding most of the I domain was obtained by polymerase chain reaction (PCR) with synthetic oligonucleotides 5′-CGGATCCCCTGATTTTCAGCTCTCAGCC-3′ and 5′-ATGCTGAAAATTTGTTCTCC-3′ as primer and human α2 cDNA (22) as template. The PCR product was subcloned into pCRII vector and then into pGEX-2T vector using *Bam*HI/*Eco*RI sites. Mutant I domain cDNA fragments were obtained by PCR amplification with mutant α2 cDNAs (36) as templates. Authenticity of the PCR product was confirmed by sequencing. The α2 portion of GST-α2I contains human α2 amino acid sequence from Ser-124 to Ile-335 (212 residues) and an extra sequence at the carboxyl terminus after Ile-335 (Ser-Arg-Ile-His-Arg-Asp) that is not present in α2.

GST-α2I fusion protein or wild type GST was prepared according to Smith and Johnson (43). Overnight culture of *Escherichia coli* DH5α containing pGEX-2T α2I plasmid was diluted 10 times into fresh LB medium. After 1 h of culture, GST fusion protein or wild type GST production was induced by adding 0.1 mM isopropyl-1-thio-β-D-galactopyranoside in culture medium and continued culture for 5 h at 37 °C. Protein was extracted from bacterial suspension in PBS (1–2% volume

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[1] The abbreviations used are: I domain, inserted/interactive domain; mAbs, monoclonal antibodies; GST, glutathione *S*-transferase; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; HBS, Hepes-buffered saline; BSA, bovine serum albumin.

of the original culture) by sonication. GST-α2I or GST was purified from the bacterial extract by glutathione-agarose (sulfur linkage) (Sigma) chromatography. GST-α2I or GST was eluted with 5 mM reduced glutathione in 50 mM Tris/HCl, pH 7.5.

Binding of GST-α2I to Immobilized Ligand—Wells of 96-well plastic plates (Immulon 2) were coated with collagen (rat tail, type I, 10 μg/ml PBS) (Collaborative Research, Bedford, MA) at 4 °C overnight. Remaining protein binding sites were blocked by incubating with 0.1% BSA (crystal) (Calbiochem) for 1 h at room temperature. GST-α2I and wild type GST were ^{125}I-labeled using iodobeads (Pierce) according to manufacturer's instructions. Free ^{125}I was removed by gel filtration on Sephadex G-25 (Pharmacia Biotech Inc.). Specific radioactivity was approximately 2.1×10^5 and 1.8×10^5 cpm/ng of protein for GST-α2I and wild type GST, respectively. ^{125}I-labeled GST-α2I and wild type GST at the indicated concentrations were incubated with collagen-coated plastic in 50 μl of Hepes-buffered saline (HBS) (10 mM Hepes, 0.15 M NaCl, pH 7.4) supplemented with 0.1% BSA and incubated for up to 3 h at room temperature. After washing with HBS with 0.1% BSA, bound protein was quantified by measuring the radioactivity on the plastic. Collagen at 10 μg/ml concentration on coated wells gives saturated binding of GST-α2I; the increase of the concentration to 100 μg/ml does not significantly increase the GST-α2I binding.

Binding of ^{125}I-labeled Collagen to GST Fusion Protein Immobilized to Glutathione-agarose—Collagen type I was ^{125}I-labeled using iodobeads as previously described (44). Free ^{125}I was removed by extensive dialysis against 0.05 M acetic acid. Specific radioactivity is approximately 2.7×10^5 cpm/mg of collagen protein. Purified GST fusion proteins were subjected to gel filtration chromatography to remove glutathione and incubated with glutathione-agarose at room temperature for 1 h in PBS. GST fusion protein-glutathione-agarose complex was washed with HBS with 0.1% BSA. The amount of protein adsorbed was determined by the difference of protein before and after incubation. The GST fusion protein-GSH-agarose complex (87 ± 9.5 μg of GST fusion protein per 10 μl of packed volume of beads) was incubated with ^{125}I-labeled collagen (7.2 μg) in HBS with 0.1% BSA (total 100 μl of incubation mixture) at room temperature for 3 h. Free and bound collagen was separated by centrifuging after loading the incubation mixture on top of 20% (w/v) sucrose in HBS with 0.1% BSA. The radioactivity in the sediment was measured.

Adhesion of CHO Cells to Collagen—Wells of 96-well microtiter plates (Immulon-2, Dynatech Labs., Inc., Chantilly, VA) were coated with type I collagen (10 μg/ml) at 4 °C overnight. The other protein binding sites were blocked by incubating with 1% (w/v) bovine serum albumin (Calbiochem) for 30 min at room temperature and washed twice with PBS (10 mM phosphate, 0.15 M NaCl, pH 7.4). Cells were harvested with 3.5 mM EDTA in PBS and washed twice with Dulbecco's modified Eagle's medium. 10^5 cells (in 100 μl of Dulbecco's modified Eagle's medium) were added to each well and incubated for 1 h at 37 °C. The wells were rinsed with PBS three times to remove unbound cells, and bound cells were quantified by assaying endogenous phosphatase activity (45).

Other Methods—Site-directed mutagenesis was carried out by unique site elimination with double-stranded vector (46).

RESULTS AND DISCUSSION

Expression of the Recombinant I Domain Fragment (GST-α2I)—A cDNA fragment containing the I domain was obtained by PCR amplification and subcloned into pGEX-2T vector. The fusion protein was expressed in *E. coli* DH5α and purified from bacterial extract by GSH affinity chromatography. The purified protein migrated as a 45-kDa protein band on SDS-polyacrylamide gel electrophoresis under reducing and nonreducing conditions (Fig. 1). We first examined the reactivity of function-blocking anti-α2 mAbs to the GST-α2I fusion protein. We previously found that the function-blocking anti-α2 mAbs (12F1, 6F1, 5E8, AA10, P1E6, P4B4, P1H5, RMAC11) recognize a small region within the I domains (36). All the mAbs listed above recognize the GST-α2I using enzyme-linked immunosorbent assay but not wild type GST. Nonfunction-blocking anti-α2 mAb HAS-3 or HAS-4 does not bind to GST-α2I (data not shown). These findings are consistent with the previous conclusion that the I domain is responsible for ligand binding (36) and suggest that the recombinant I domain may be properly folded and probably maintains an overall conformation similar to that found in the whole transmembrane receptor.

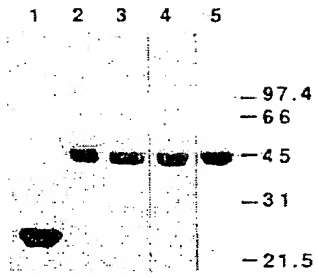

FIG. 1. **Glutathione *S*-transferase α2 I domain fusion proteins (wild type and mutants) and wild type GST.** A cDNA fragment of α2 subunit (residues 124–335, wild type and mutants) was obtained by PCR amplification and subcloned into pGEX-2T vector (43). The glutathione *S*-transferase α2 I domain fusion proteins and wild type GST were synthesized in *E. coli*, extracted from bacteria, and purified by affinity chromatography on glutathione-agarose. Bound protein was eluted by 5 mM reduced glutathione in 50 mM Tris/HCl, pH 7.5. Eluted materials were analyzed by SDS-gel electrophoresis using 10% gel and staining with Coomassie Blue. *Lane 1*, wild type GST (M_r, about 23,000); *lane 2*, GST-α2 I domain (M_r, about 45,000); *lane 3*, GST-α2I(D151A) mutant; *lane 4*, GST-α2I(D254A) mutant; *lane 5*, GST-α2I(T221A) mutant. The difference (22,000) between GST and GST fusion proteins corresponds to 212 amino acid residues of the I domain.

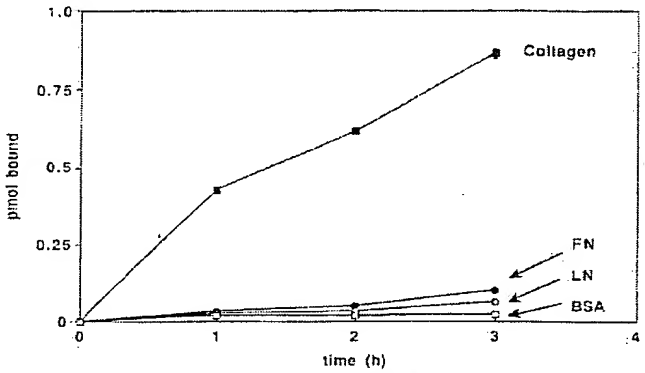

FIG. 2. **Time course of binding of ^{125}I-labeled GST-α2I fusion protein to immobilized substrates.** GST-α2I fusion protein was labeled with ^{125}I using iodobeads (Pierce) following instructions of the manufacturer. Concentration of substrates used for coating was 10 μg/ml PBS. ^{125}I-GST-α2I (50 nM) was incubated with substrates in 50 μl of HBS (10 mM Hepes, 0.15 M NaCl, pH 7.4) supplemented with 0.1% BSA in microtiter wells at room temperature up to 3 h. Data are shown as means ± S.D. of triplicate experiments. *FN*, fibronectin; *LN*, laminin.

Binding of the Recombinant I Domain Fragment to Immobilized Collagen Type I—We studied the interaction between ^{125}I-labeled GST-α2I and collagen immobilized on plastic. Fig. 2 shows that the ^{125}I-labeled GST-α2I fusion protein interacts with collagen but not with BSA. Control ^{125}I-labeled wild type GST did not show significant binding to either collagen or BSA. The binding of GST-α2I is in proportion to the incubation time (at least to 3 h). The GST-α2I fusion protein did not show significant affinity to fibronectin, laminin (Fig. 2), fibrinogen, and vitronectin (at 10 μg/ml coating concentrations). ^{125}I-GST-α2I binding to collagen was almost completely blocked by the anti-α2 mAbs that block collagen binding to α2β1 (6F1 and 5E8 at 10 μg/ml concentration) and by a 50-fold molar excess of cold GST-α2I but not by the anti-α2 mAb that does not block collagen binding to α2β1 (12F1 at 10 μg/ml concentration). These data indicate that the GST-α2I binding to collagen is specific. The binding of GST-α2I as a function of collagen concentration used for coating wells was examined. Collagen concentration at 10 μg/ml on coating wells gives maximal binding of GST-α2I (data not shown).

Fig. 3 shows the binding of GST-α2I to collagen as a function of increasing concentration of GST-α2I. Nonspecific binding of ^{125}I-GST-α2I obtained in the presence of a 50-fold molar excess of cold GST-α2I was less than 10% of the total binding and was subtracted from the total binding. The concentration of GST-α2I that gave half-maximal binding is calculated as 5×10^{-7} M.

The data indicate that the recombinant I domain fragment specifically interacts with collagen. The specificity of the binding was established by showing that 1) GST-α2I does not significantly bind to BSA as negative control for ligand, 2) function-blocking anti-α2 mAbs block the binding, and 3) excess cold GST-α2I blocks the binding of ^{125}I-GST-α2I to collagen. The concentration (5×10^{-7} M) of GST-α2I that gave half-maximal binding to collagen is comparable with the K_d values for fibronectin in binding to fibroblasts or platelets (8×10^{-7} M and 2.2×10^{-7} M, respectively) (47, 48). K_d values for α2β1 integrin-collagen interactions have not been reported. These results indicate that the I domain portion of α2 is sufficient for high affinity collagen binding. There is a possibility that distinct binding sites on α2β1 are involved for collagen and laminin since GST-α2I did not bind to laminin.

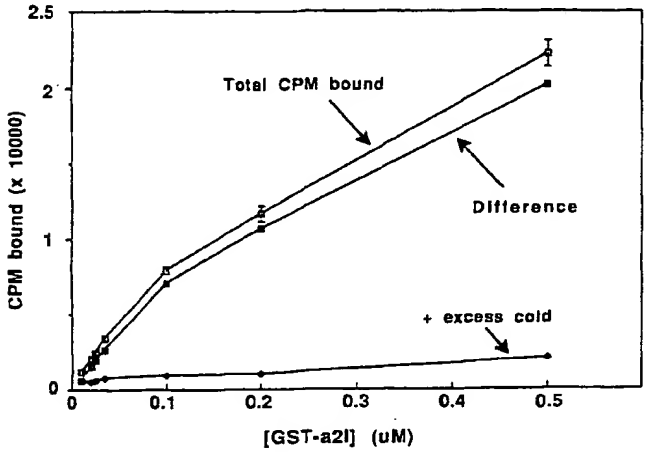

FIG. 3. **Binding of GST-α2I to collagen as a function of GST-α2I concentration.** ^{125}I-GST-α2I at increasing concentrations was incubated with collagen-coated plastic. Incubation was carried out at room temperature for 1 h. Collagen type I (10 μg/ml PBS) was used for coating. To obtain nonspecific binding, a 50-fold molar excess of cold GST-α2I was added. Data are shown as means ± S.D. of triplicate experiments. The concentration of GST-α2I that gave half-maximal binding is calculated as 5×10^{-7} M using a double reciprocal plot.

Divalent Cations and EDTA Do Not Affect the Collagen Binding to the Recombinant I Domain Fragment—Ligand binding characteristics of GST-α2I were further analyzed to determine if the recombinant I domain has the binding characteristics of intact α2β1 integrin. Mg^{2+} or Mn^{2+} are required for and Ca^{2+} has an inhibitory effect on collagen binding of α2β1 integrin. We examined the divalent cation requirement for the GST-α2I-collagen interaction. Fig. 4 shows that the addition of exogenous Mg^{2+}, Mn^{2+}, or Ca^{2+} has no effect on the collagen-GST-α2I interaction. Notably, EDTA (up to 5 mM) does not inhibit the interaction. These findings strongly suggest that the actual collagen binding is not divalent cation-dependent, even though α2β1 whole heterodimer requires Mg^{2+} or Mn^{2+} for ligand binding. These data indicate that Mg^{2+} or Mn^{2+} may be involved in the maintenance of an active conformation of the α2β1 heterodimer (or in regulating the accessibility of the ligand binding sites to collagen) but not in the actual collagen-I domain interaction. It is not likely that divalent metals are so firmly associated with the I domain that the association is resistant to EDTA treatment because 1 mM EDTA completely blocks collagen binding to α2β1 heterodimer.

Mutations of Asp-151 and Asp-254 That Completely Block Ligand Binding of α2β1 Do Not Completely Block Collagen Binding to the Recombinant I Domain Fragment—Divalent cation binding to the I domain of αM has been reported, and Asp-140 and Asp-242 appear to be critical since mutation of these residues to Ala blocks divalent cation binding to recombinant αM I domain fusion protein (37). Mutations of these residues block binding of iC3b to integrin αMβ2 probably due to blocking of divalent cation binding to the αM I domain (37). We already reported that mutations of Asp-151 and Asp-254 of α2 corresponding to Asp-140 and Asp-242 of αM completely block the binding of α2β1 to collagen (36), suggesting similar ligand binding mechanisms between α2 and αM I domains.

We examined the effects of mutating Asp-151 and Asp-254 to Ala (designated D151A and D254A, respectively) in the α2I fusion protein. α2I fusion proteins with D151A and D254A (designated α2I(D151A) and α2I(D254A), respectively) were prepared and tested for collagen binding. Fig. 5 shows that D151A mutation has no effect on collagen binding, while the D254A mutation inhibits partially. These findings show that Asp-151

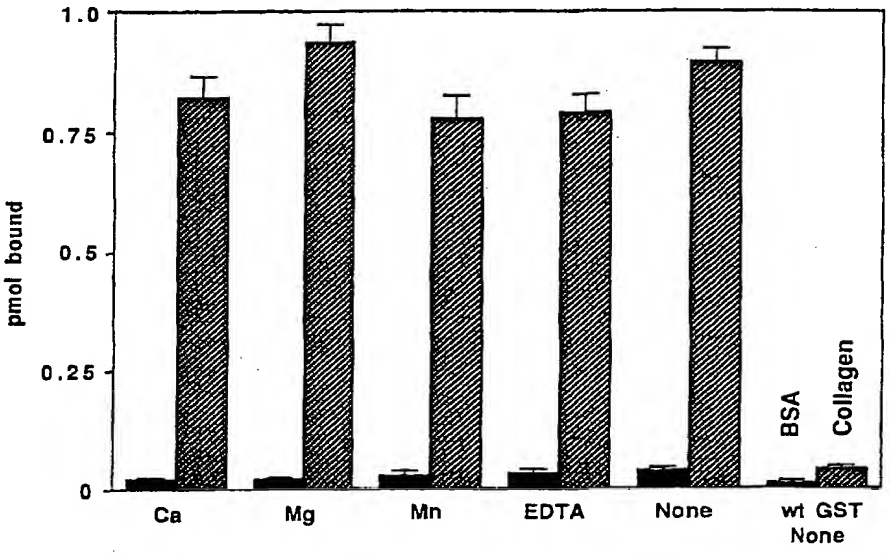

FIG. 4. **Divalent cations or EDTA do not affect GST-α2I binding to collagen type I.** Ca^{2+}, Mg^{2+}, Mn^{2+}, and EDTA (1 mM) were added to the incubation mixture (50 nM ^{125}I-GST-α2I in 50 μl of HBS supplemented with 0.1% BSA). After incubation for 3 h at room temperature, bound GST-α2I was determined. Data are shown as means ± S.D. of triplicate experiments. ^{125}I-labeled wild type GST does not bind to collagen. BSA does not support binding of GST-α2I.

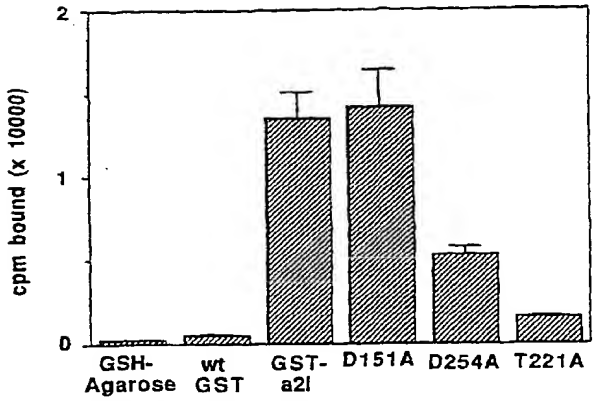

FIG. 5. **The effects of D151A, D254A, and T221A mutations on collagen binding function of GST-α2I fusion protein.** GST-α2I fusion proteins with D151A, D254A, and T221A mutations were prepared as described under "Experimental Procedures." The fusion proteins were coupled to glutathione-agarose and incubated with ^{125}I-labeled collagen for 3 h at room temperature. The radioactivity bound to the fusion proteins was measured. Data are shown as means ± S.D. of triplicate experiments. *wt*, wild type.

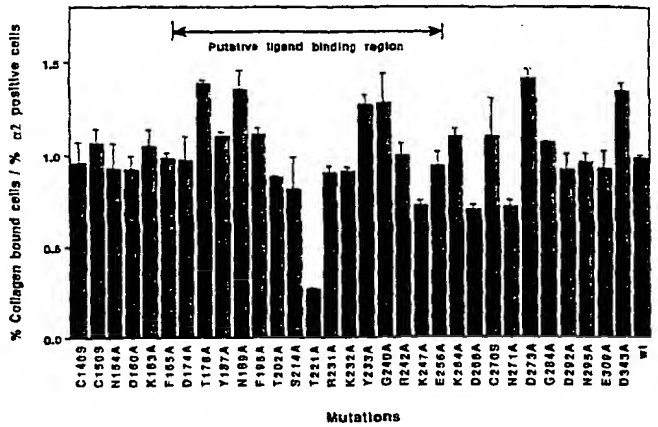

FIG. 6. **The effects of substitution of amino acid residues of the I domain on collagen binding of α2β1 on CHO cells.** Amino acid residues were substituted with Ala except for Cys, which was replaced with Ser. CHO cells stably expressing wild type (*wt*) or mutant α2 were analyzed for expression of human α2 using flow cytometry with mAb 5E8 and for adhesion to collagen (type I). Cell adhesion to collagen was carried out in triplicate experiments. The data are expressed as percentage of collagen bound cells per the percentage of α2 positive cells. CHO cells expressing α2(T221A) show background level adhesion to collagen (approximately 10%), despite the fact that approximately 40% of cells are 5E8 positive. mAb 12F1 instead of 5E8 was used for α2(R242A) mutant.

(and probably Asp-254 as well) are not critical in collagen binding to the α2I fusion protein while they are critical for the intact receptor. The data suggest that Asp-151 is probably not involved in the actual binding to collagen, but in the regulation of binding to α2β1, for example, via effects on accessibility of ligands or maintenance of active conformation of the true ligand binding site. These effects are likely to be mediated through binding of divalent cations to the I domain. We do not rule out the possibility that Asp-254 is involved in the direct ligand binding since D254A mutation partially blocks the binding of GST-α2I to collagen. The ligand binding functions of the wild type and mutant αM I domain fusion proteins have not been reported.

Alanine-scanning Mutagenesis of the I Domain (Thr-221 within the Putative Ligand Binding Site Is Critical for Collagen Binding)—We previously identified a small region within the I domain (residues 173–259) as the putative ligand binding sites, based on the fact that it is recognized by function-blocking anti-α2 mAbs (36). We introduced point mutations (to Ala) within and around the putative ligand binding sites. Mutant α2 cDNAs were used for transfection of CHO cells and selected for G418 resistance. Most of the mutant α2 constructs were stably expressed on CHO cells, except for D145A and D259A mutants, which were not expressed at all. Fig. 6 shows collagen binding of the α2 mutants expressed as percentage of cells adherent to collagen per percentage of human α2 positive cells (normalized collagen binding) with the population of G418-resistant CHO cells. In most cases, percentage of α2 positive cells and percentage of adherent cells to collagen correlate (normalized collagen binding between 0.7 and 1.3), suggesting most mutations do not have significant effects on the collagen binding of α2β1. Only T221A mutant shows very low normalized collagen binding (approximately 0.25). α2(T221A) is stably expressed on CHO cells and reacts with all the anti-α2 mAbs tested (AA10, P1H5, 5E8, P4B4, and RMAC11). We further tested the adhesion properties of T221A mutant α2 with cloned α2(T221A) CHO cells. Table I shows that the T221A mutation of α2 completely blocks adhesion of CHO cells to collagen with homogeneous α2(T221A) CHO cells, although the nearby D219A mutation does not affect the adhesion to collagen.

We tested the collagen binding of GST-α2I fusion protein with T221A mutation (α2I-T221A). α2I-T221A fusion protein binds to collagen only weakly (Fig. 5). The data suggest that

TABLE I

The effect of D219A and T221A mutations on collagen binding.

Numbers in column 2–4 represent percentage of bound cells (± S.D.) to plastic coated with proteins indicated. Percentage of positive cells and mean fluorescence intensity with control mouse IgG to each cell line was 0.6–2.2% and 2.7–4.3, respectively (background level). The reactivity of the mAbs (6F1, P1H5, RMAC11, 5E8, P4B4, AA10) is not affected by these mutations.

	10 μg/ml collagen	10 μg/ml fibronectin	Bovine serum albumin only	% of positive cells with mAb 5E8	Mean fluorescent intensity
CHO cells	11.9 ± 0.8	93.6 ± 2.5	5.5 ± 0.9	3.5	4.2
T221A α2	10.2 ± 0.6	82.1 ± 3.5	2.5 ± 0.1	98.5	237
D219A α2	92.0 ± 2.7	99.1 ± 1.0	3.3 ± 0.3	98.7	296
Wild type α2	80.6 ± 1.6	92.5 ± 1.4	2.5 ± 0.8	82.1	56

Thr-221 (and probably a region containing Thr-221) is critical for collagen binding both for α2β1 on CHO cells and for the purified GST-α2I fusion protein in contrast to Asp-151 and Asp-254, which are critical for α2β1 but not for the I domain fragment. The data are consistent with the previous conclusions that a small region of the I domain (173–259) contains the ligand binding sites (36). Since Thr-221 is conserved among I domains (22), it is possible that this residue is critical for ligand-I domain interactions in general. It will be interesting to examine if the corresponding Thr residues of other I domain-containing proteins (*e.g.* αlβ1, αLβ2, αMβ2, αXβ2, von Willebrand factor, cartilage matrix protein, αEβ7) are involved in ligand binding and if divalent cations are required for the binding of ligand to the other I domains.

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The Role of the I Domain in Ligand Binding of the Human Integrin $\alpha_1\beta_1$*

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We report here the analysis of potential ligand binding domains within the human integrin α_1 subunit, a known collagen/laminin receptor. This integrin is effectively blocked by the mouse monoclonal antibody 1B3.1. A truncated version of the α1 subunit lacking the NH_2-terminal half of the extracellular domain is not recognized by monoclonal antibody 1B3.1. Furthermore, we have isolated a cDNA containing the I domain from chicken α_1 bearing significant homology to the human and rat α_1 sequences. Replacing the human I domain with its chicken counterpart led to the surface expression of a functional heterodimer with endogenous mouse β_1 on NIH 3T3 cells. However, 1B3.1 does not bind to the chicken/human chimera, demonstrating that the human α_1 I domain is required for epitope recognition. Mutation of Asp^{253} within the I domain to alanine resulted in surface expression of an αβ heterodimer recognized by 1B3.1 but with markedly reduced binding to collagen IV or laminin. Since a previously reported mutation of a homologous Asp in the Mac-1 I domain has similar consequences, these results suggest a central role for the I domain in ligand recognition for all integrin α subunits containing this domain.

Integrins, a family of cell surface receptors, have a prominent role in cell-cell and cell-matrix adhesion events important for processes such as embryonic development and morphogenesis, hemostasis, and thrombosis, wound healing, metastasis, and leukocyte helper and killer function. Integrins recognize a wide variety of ligands including extracellular matrix proteins, serum proteins, and counter-receptors on other cells.

Integrins are expressed as heterodimers composed of noncovalently associated α and β subunits. At least 15 distinct α and 8 distinct β subunits have been identified and the dimers formed between them are divided into classes based on their β subunit (1). The α subunits, which have a repeating modular structure, can be separated into two groups, with the exception of α_4 (2). The first type of α subunit has a post-translational intrachain cleavage and four discrete metal binding domains, each contained within a repeat. The other form has an inserted or I domain of 210 amino acids (3), found between repeats 3 and 4, which interrupts the modular structure, and has three discrete divalent cation binding sites within repeats 5–7. There are five α subunits, α_1, α_2, α_{LFA-1}, α_{Mac-1}, and α_{p150} which belong to this distinct subset of integrins.

Most integrin receptors can interact with several different ligands while individual ligands can be recognized by various heterodimers. Fibronectin (FN),[1] for example, is a ligand for at least 8 different integrin receptors. The specificity of receptor-ligand interaction is provided by both the α and the β subunits, as shown by the α_v containing integrins which recognize different ligands depending on the β subunit present (4–6). Further ligand recognition complexity is provided through the ability of cells to regulate the activation status of their integrins through inside-out signaling (7–9).

Integrin domains involved in ligand recognition have been found in both α and β subunits and there is a relationship between ligand and divalent cation binding sites. The role of the β subunit in ligand binding has been demonstrated by cross-linking RGD peptides (the major cell binding motif in FN) to a region spanning residues 109–170 in the β_3 subunit of the integrin gpIIb/IIIa (10, 11). The first 20 amino acids of this region are particularly interesting because of the high degree of conservation among β subunits. In addition, this region may also contribute to divalent cation binding. Loftus *et al.* (12) has shown that mutations in this region which would disrupt β_3 cation binding have dramatic effects on ligand binding as well. Furthermore, Takada *et al.* (13) have mutated the corresponding aspartate residue in the β_1 subunit, perturbing ligand binding. Last, epitope mapping of blocking antibodies to the β_1 subunit show that the region bound by antibody is very close to the putative RGD binding domain (14).

Cation binding domains within α subunits have been implicated in ligand binding in several cases. Studies on the α_{IIb} integrin subunit showed that peptides derived from the second metal binding domain (within repeat five) could compete with the intact receptor for fibrinogen binding and that an aspartate residue was required for this effect (15). Similarly, mutation of cation binding domains within $\alpha_4\beta_1$ had significant effects on the binding of this integrin to its ligands (16).

Recent work has suggested that these divalent cation binding regions are not exclusively responsible for ligand recognition by α subunits. A number of experiments strongly implicate the I domain in ligand recognition. Very recently, the role of the I domain in binding divalent cations has been demonstrated. Expression of this domain as a bacterial fusion protein led to

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U10114.

‡ Recipient of a long term fellowship from The Human Frontier Science Organization no. LT 653.

¶ Recipient of a junior faculty award from the American Cancer Society. To whom correspondence should be addressed: Dept. of Pathology, 630 W. 168th St., Black Bldg. 1427, New York, NY 10032. Tel.: 212-305-2017; Fax: 212-305-5498.

[1] The abbreviations used are: FN, fibronectin; Col IV, type IV collagen; LN, laminin; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; mAb, monoclonal antibody.

the recovery of a protein fragment capable of binding several divalent cations (17). In addition, mutation of an aspartate residue within the I domain of full length $\alpha_{Mac\text{-}1}$ led to a receptor with impaired ligand binding properties. Furthermore, Diamond *et al.* (18) has shown that a large number of α subunit blocking antibodies bind to the I domain of $\alpha_{Mac\text{-}1}$.

The I domain has been implicated in collagen binding by integrins based on sequence homology of this region with collagen binding domains of Von Willebrand factor (19). In this study, we identified the domains of the human α_1 subunit involved in the function of the $\alpha_1\beta_1$ integrin as a receptor for collagen IV (Col IV) and laminin (LN). We then demonstrated that the epitope of a function blocking antibody, 1B3.1, can be mapped to the I domain and that mutagenesis of an aspartate residue within the I domain led to a marked reduction in ligand binding.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Plasmid Construction—Restriction enzymes and polynucleotide kinase were purchased from New England Biolabs, T4 DNA ligase from Life Technologies, Inc., and *Taq* polymerase from Boehringer Mannheim. Standard recombinant DNA methods were used (20). Oligonucleotides were purchased from Operon Technologies (Alameda, CA). The construction of the α_1NΔ538 truncated form of the α_1 subunit is described elsewhere.[2]

Isolation of Chicken I Domain Sequence—Total RNA was prepared from chicken gizzards from day 12 embryos as described elsewhere (21). One μg of poly(A)$^+$ mRNA was isolated by elution from oligo(dT)-cellulose (Pharmacia Biotech Inc.). This mRNA was used to synthesize randomly primed cDNA by standard methods (22), and treated with RNase prior to PCR using the following oligonucleotides: sense, 5′-AA(A/G)-GA(A/G)-AA(C/T)-ATG-ACI-TT(C/T)-GG-3′; antisense, 5′-CCI-AIC-ATI-ACC-CA(G/A)-TC(T/C)-TG-3′. The resulting PCR fragment of 853 base pairs was cloned into the *Sma*I site of pBluescript (Stratagene, La Jolla, CA) and sequenced using Sequenase (U.S. Biochemical Corp., Cleveland, OH). The portion of the cDNA corresponding to the chicken I domain was exchanged for the human I domain using overlapping PCR (23). The oligonucleotide for the sense strand is: 5′-CCT-GTA-CAA-GAA(human)-TGT-AAA-ACC-CAG-TTG-GA(chicken)-3′; for the antisense strand, 5′-GCC-AGT-CTG-AGA(human)-CAT-TTC-CAT-TTC-AAA-TGA(chicken)-3′. The linked fragment was cut with *Acc*I and *Apa*I and ligated into the human α_1 cDNA to reconstitute a full-length cDNA in pLEN. The amino acid sequence of the transition points within the chimeric α_1 subunit and the position number of the mature protein (according to Briesewitz *et al.* (24)) for the human α_1 sequence and Fig. 2*A* of this article for the underlined chicken sequence) is: PVQE^{138}C^{52}KTQLD. . . . FEMEM^{261}S^{350}QTGF.

Construction of I Domain Point Mutation—A point mutation, G^{848}AT to G^{848}CT, leading to an Asp^{253} → Ala amino acid substitution within the human α_1 I domain was introduced using oligonucleotides and overlapping PCR, cloned into the full length pBluescript α_1 cDNA via *Apa*I and *Acc*I and then cloned into the expression vector pLEN. The fidelity of all regions amplified by polymerase chain reaction was verified by sequencing.

DNA Transfections—NIH 3T3 cells were maintained in DMEM with 10% calf serum 5 × 10^5 cells plated the previous day were co-transfected with 20 μg of integrin plasmid and 2 μg of pSVneo (25) as a calcium phosphate precipitate as previously described (26). After 3 days, the cells were split 1:20 into DMEM supplemented with 10% calf serum and 1.0 mg/ml G418 (Life Technologies, Inc.). After 10–14 days, G418-resistant clones were isolated and screened by immunoprecipitation.

Antibodies—Rabbit anti-β_1 cytoplasmic peptide serum was prepared as described elsewhere (27). Monoclonal anti-human α_1 (TS2/7) was a gift of T. Springer (Harvard Medical School). Monoclonal anti-human α_1 1B3.1 was prepared as described elsewhere (28).

Radiolabeling and Immunoprecipitation—Cells were labeled with Na^{125}I (NEN-DuPont) and lactoperoxidase as described previously (29). 10^7 cells and 1 mCi/ml were used per experiment. The cells were washed three times with PBS and extracted with 0.5% Nonidet P-40 and immunoprecipitation as described elsewhere (27).

SDS-PAGE was performed according to Laemmli (30). Separation gels were 7.0% acrylamide with a 3% stacking gel. Samples were prepared in sample buffer (5% SDS, 100 mM Tris-HCl, pH 6.8, 10 mM EDTA, 10% glycerol, and bromphenol blue) and boiled for 3 min.

Affinity Chromatography—The CB3 fragment of human collagen type IV was prepared and coupled to Sepharose as described elsewhere (31). 3T3 cell lines were surface labeled with ^{125}I. Cell extracts were prepared using 50 mM β-octyl glucoside in 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$ (TMM). After a 10 min incubation on ice, insoluble material was removed by centrifugation at 10,000 × *g* for 20 min. Four-fifths of the resulting supernatant was incubated batchwise with 100 μl of beads (CB3-Sepharose) for 1 h at 4 °C with end-over-end mixing. The beads were recovered by sedimentation and washed four times with 50 mM β-octyl glucoside in TMM, followed by elution with 1 ml of 50 mM β-octyl glucoside in TMM containing 20 mM EDTA. This eluate was immunoprecipitated with rabbit anti-β_1 cytoplasmic peptide serum and compared with an immunoprecipitate of 1/10 of the cleared extract, followed by SDS-PAGE.

Cell Adhesion Assays—Adhesion assays were done essentially as previously described (32). Adhesive proteins were diluted in 0.1 M acetic acid (Col IV) or water (FN, LN) and dried down in microtiter wells at room temperature. After washing with PBS, remaining protein binding sites were blocked with heat treated bovine serum albumin (1% in PBS). Cells were suspended in serum-free DMEM (4–5 × 10^5/ml) and incubated for 30 min at 37 °C. Nonadherent cells were removed by washing with PBS, and bound cells were fixed with 70% ethanol and stained with crystal violet (0.1% in water). Excess stain was removed with water. Bound color was dissolved with Triton X-100 (0.2% in water), and the optical density was read at 595 nm.

For inhibition assays, cells were preincubated for 10 min at room temperature with mouse anti-human α_1 mAb 1B3.1 (28) diluted 1:200 in DMEM, or with 5 mM EDTA in DMEM and then transferred to wells coated with LN or Col IV (see figure legends for respective amounts). After 30 min at 37 °C, nonadherent cells were removed and bound cells fixed and detected as described above. All experiments were done in triplicate.

RESULTS

Epitope Mapping of Function-blocking Anti-α_1 Monoclonal Antibody—We began our investigation of putative ligand binding domains within the human α_1 subunit by mapping the epitope of the function blocking mAb 1B3.1 (28). This monoclonal antibody completely blocks α_1-dependent adhesion to Col IV and LN (33), the primary ligands for $\alpha_1\beta_1$. We had previously developed two NIH 3T3 mouse fibroblast cell lines which were useful for this analysis. One NIH 3T3 line, HA1, expresses the wild type human α_1 subunit in a complex with the endogenous mouse β_1 subunit (24), while the other 3T3 cell line, called α_1NΔ538cyt, expresses a truncated form of human α_1, which is expressed as a monomer, and is missing the amino-terminal half of the extracellular domain.[2] We compared the reactivity of the blocking antibody 1B3.1 and the non-blocking antibody TS2/7 toward the truncated and full length human α_1 subunits expressed in 3T3 cells. Both cell lines were surface labeled with Na^{125}I and a detergent extract was immunoprecipitated with rabbit anti-β_1 cytoplasmic peptide serum (Fig. 1, *a* and *d*) and the two mouse anti-human α_1 monoclonal antibodies (Fig. 1, *b* and *e*, TS2/7; *c* and *f*, 1B3.1). HA1 cells express the human α_1 subunit in a dimer with endogenous mouse β_1 as shown by co-precipitation of β_1 with α_1 specific antibodies (Fig. 1, *b* and *c*). α_1NΔ538cyt cells express the truncated form of α_1 on the surface, precipitated with TS2/7 (Fig. 1*e*), but β_1 is not coprecipitated and precipitation of the β_1 integrins does not show the truncated α_1 chain (Fig. 1*d*), indicating that this subunit does not dimerize with the endogenous mouse β_1 subunit.[2] The non-blocking antibody TS2/7 reacts equally well with the full-length and truncated forms of α_1 (Fig. 1, *b* and *e*), indicating that the recognized epitope is within the COOH-terminal extracellular part of α_1. In contrast, the blocking mAb 1B3.1 does not recognize the truncated form of α_1, while effectively recovering intact $\alpha_1\beta_1$ (Fig. 1, *c* and *f*). These results strongly suggest that the region recognized by this blocking antibody is within the NH_2-terminal portion of the extracellular domain.

In cell adhesion assays (not shown) we demonstrated that

[2] L. Smilenov, R. Briesewitz, and E. E. Marcantonio, manuscript in preparation.

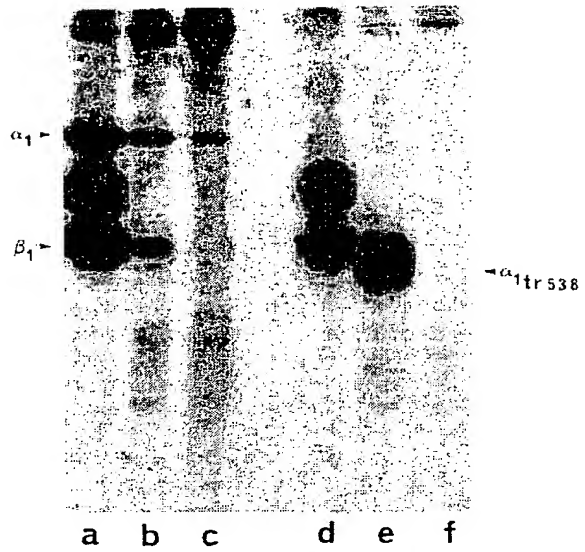

FIG. 1. **Expression of full length and truncated human α_1 subunits in mouse NIH 3T3 cells.** Extracts of ^{125}I surface labeled HA1 (*a–c*) or α1NΔ538cyt (*d* and *e*) were incubated with rabbit anti-β_1 cytoplasmic peptide serum (*a* and *d*), mouse monoclonal anti-human α_1 antibody TS2/7 (*b* and *e*), or mouse monoclonal anti-human α_1 antibody 1B3.1 (*c* and *f*). Immunoprecipitates were recovered by the use of protein A-Sepharose (*a* and *d*) or goat anti-mouse agarose (*b*, *c*, *e*, and *f*), followed by analysis using SDS-PAGE and autoradiography. A heterodimer consisting of human α_1 and mouse β_1 was recovered from HA1 cells. The truncated form of α_1 from α1NΔ538cyt cells reacts with TS2/7 but does not dimerize with mouse β_1. mAB 1B3.1 does not react with the truncated α_1 subunit.

cells expressing the truncated α_1 chain bind poorly to Col IV and LN, similar to the parental 3T3 fibroblasts. This property is in contrast to HA1 cells, which adhere to Col IV in an α_1-dependent manner (33).

Isolation of the Chicken α_1 I Domain—The region of α_1 which is missing in the α_1NΔ538 construct includes repeats 1–4, the I domain, repeat 5, and the α_1-specific cystine-rich region. Since there is evidence from studies of the β_2 integrins that the I domain may be recognized by ligand blocking antibodies (18), we decided to use the I domain from chicken α_1 to determine if the presence of the human α_1 I domain was required for recognition by 1B3. To examine this possibility, we isolated a partial cDNA for chicken α_1 from mRNA using reverse transcription followed by PCR using degenerate oligonucleotides derived from the human α_1 sequence (see "Experimental Procedures" for details). This cDNA was cloned and sequenced.

We compared the deduced chicken α_1 protein sequence with the published sequences of the human (24) and rat α_1 protein (34) and found that 83% of the amino acids are identical. Fig. 2 shows the sequence of the partial chicken cDNA clone and a comparison with the corresponding human α_1 sequence (Fig. 2*B*). Overall the sequences are significantly homologous with only rare nonconservative substitutions.

As described under "Experimental Procedures," we exchanged the chicken I domain for the corresponding region in the human α_1 sequence and reconstituted a full-length α_1 subunit. This chimeric cDNA in an expression vector was co-transfected into NIH 3T3 mouse fibroblasts with a plasmid for neomycin resistance. A 3T3 cell line (called cID) expressing the chimeric protein was isolated and compared to HA1 cells expressing the wild type human α_1 subunit. Both lines were surface labeled with Na^{125}I and detergent extracts were immunoprecipitated with rabbit anti-β_1 cytoplasmic peptide serum (Fig. 3, *c* and *f*) and mouse monoclonal TS2/7 (Fig. 3, *a* and *d*).

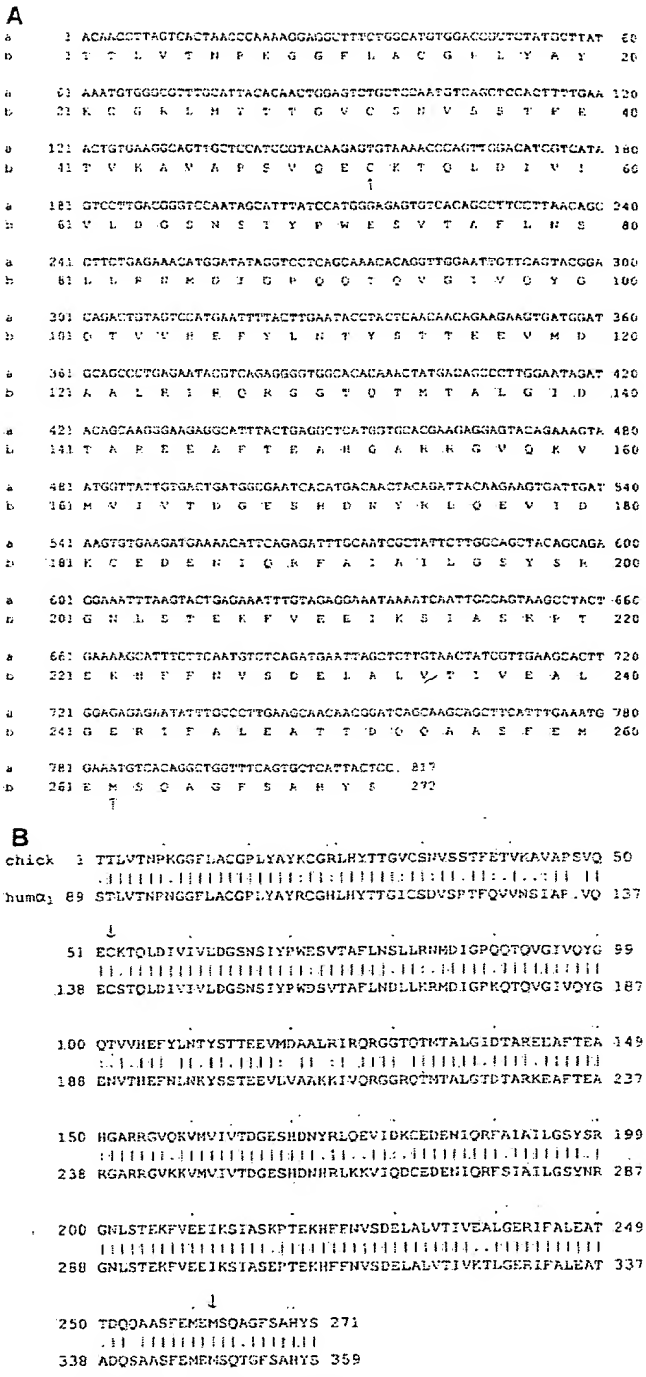

FIG. 2. *A*, nucleotide sequence of chicken α_1 I-domain cDNA and its translated amino acid sequence. The isolated cDNA sequence is shown in *line a* and the translated amino acid sequence is shown in the one-letter code in *line b*. The *arrows* depict the start (Cys^{52}) and the end (Met^{262}) of the chicken sequence in the chimeric α_1 subunit. *B*, Alignment of human α_1 and chicken α_1 I domain amino acids. The chicken α_1 amino acid sequence is compared with the human α_1 sequence using BESTFIT program in the GCG package. Identical residues are connected by *vertical lines*. The amino acid position of the mature human α_1 is numbered according to Briesewitz *et al.* (24).

The results show that the chimeric α_1 subunit, like its wild type counterpart, is expressed on the cell surface as a heterodimer with endogenous mouse β_1. In contrast, immunoprecipitation

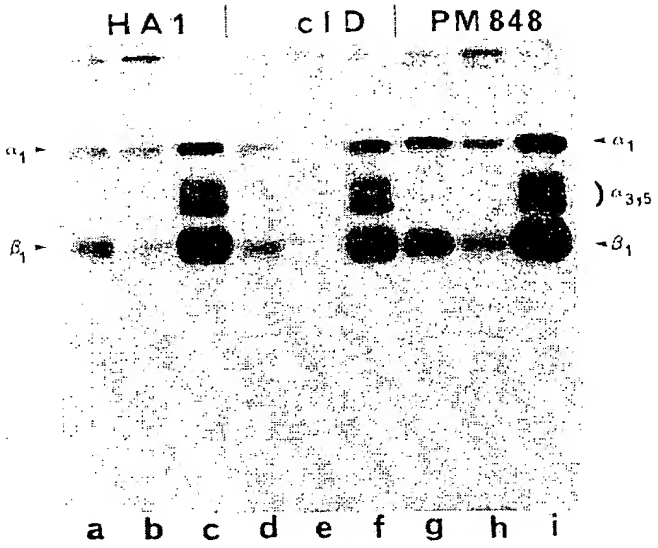

FIG. 3. **Expression of wild type and mutant human α_1 subunits in mouse NIH 3T3 cells.** Extracts of ^{125}I surface labeled HA1 (*a–c*), cID (*d* and *e*), or PM848 cells (*g–i*) were incubated with mouse monoclonal anti-human α_1 antibody TS2/7 (*a*, *d*, and *g*), mouse monoclonal anti-human α_1 antibody 1B3.1 (*b*, *e*, and *h*) or rabbit anti-β_1 cytoplasmic peptide serum (*c*, *f*, and *i*). Immunoprecipitates were recovered by the use of protein A-Sepharose (*c*, *f*, and *i*) or goat anti-mouse agarose (*a*, *b*, *d*, *e*, *g*, and *h*), followed by analysis using SDS-PAGE and autoradiography. A heterodimer consisting of human α_1 and mouse β_1 is recovered from all cell lines by anti-β_1 and anti α_1 TS2/7. The monoclonal anti-human α_1 antibody 1B3.1 does not react with the chimeric α_1 subunit in cID cells.

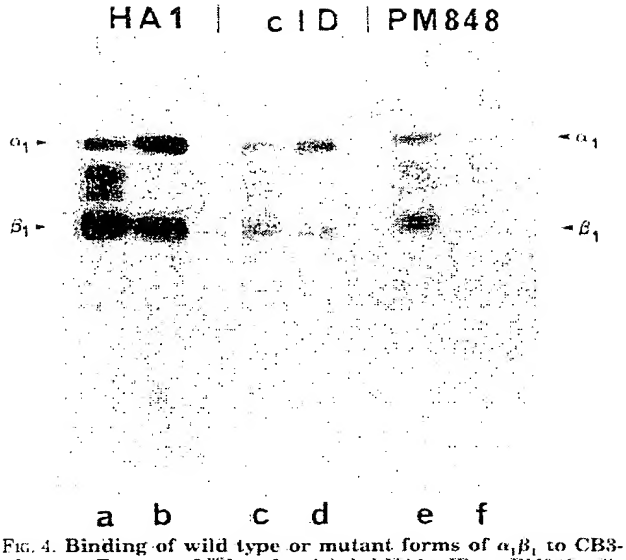

FIG. 4. **Binding of wild type or mutant forms of $\alpha_1\beta_1$ to CB3-Sepharose.** Extracts of ^{125}I surface labeled HA1, cID, or PM848 cells were prepared using β-octyl glucoside in TMM buffer. One-tenth of the extracts were incubated with rabbit anti-β_1 cytoplasmic peptide serum (*lanes a*, *c*, and *e*). Four-fifths of the extracts were incubated with CB3-Sepharose, washed, and eluted with TMM buffer containing 20 mM EDTA. These samples were then immunoprecipitated using rabbit anti-β_1 cytoplasmic peptide serum. Immunoprecipitates were recovered by the use of protein A-Sepharose and analyzed by SDS-PAGE and autoradiography. $\alpha_1\beta_1$ from HA1 and cID binds specifically to CB3-Sepharose. No binding of $\alpha_1\beta_1$ from PM848 cells was observed.

with the blocking antibody 1B3.1 (Fig. 3, *b* and *e*) selectively recovers the wild type α_1 subunit, while failing to recognize the chimeric α subunit containing the chicken I domain sequence. These experiments demonstrate the requirement of the human I domain for epitope recognition of the function blocking antibody 1B3.1.

I Domain Point Mutation—Recently, Michishita *et al.* (17) have shown that a metal binding site is contained within the I domain of the integrin Mac-1 (CD11b) and that mutations of two aspartate residues within that domain abolished ligand binding. We decided to test the role of one of the homologous aspartate residues contained within the I domain of α_1. We constructed a human α_1 cDNA where the Asp^{253} residue was changed to an Ala residue. This mutant cDNA in an expression vector was co-transfected with a neomycin resistance gene in NIH-3T3 cells. A cell line, called PM848, was isolated and characterized. PM848 cells were surface labeled with ^{125}I and a detergent extract was immunoprecipitated with rabbit anti-β_1 cytoplasmic peptide serum (Fig. 3*i*) and the mouse monoclonal antibodies TS2/7 (Fig. 3*g*) and 1B3.1 (Fig. 3*h*). The results show that the mutant α subunit is expressed on the surface in complex with endogenous mouse β_1. Thus, the change of a charged amino acid (aspartate) to alanine does not interfere with the ability to dimerize with endogenous β_1. Immunoprecipitation with anti β_1 shows a comparable expression of the human α subunit and the endogenous mouse α subunits in all three cell lines analyzed (Fig. 3, *HA1*, *cID*, and *PM848*).

Ligand Binding by Mutant α Subunits—To test the ligand binding capacity of the chimeric and mutant α_1 subunits, we performed affinity chromatography experiments, and compared the results with those using the wild type α_1 from HA1 cells. We used the cell binding fragment CB3 of type IV collagen (31), covalently coupled to Sepharose. This fragment shows high affinity interaction with both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins (35).

When detergent extracts of surface labeled HA1 or cID cells were incubated with CB3-Sepharose, we recovered $\alpha_1\beta_1$ complexes after elution with EDTA. Fig. 4 shows the results of immunoprecipitation experiments of cell extracts (Fig. 4, *a* and *c*) and EDTA eluates (Fig. 4, *b* and *d*) with the rabbit anti-β_1 cytoplasmic peptide serum. $\alpha_1\beta_1$ from HA1 and cID bound in a cation-dependent manner to the CB3-Sepharose. No endogenous mouse α subunits were detected. In contrast, when extracts from PM848 cells were used, no detectable $\alpha_1\beta_1$ complexes were seen in the EDTA eluate from from CB3-Sepharose (Fig. 4*f*), although comparable amounts of $\alpha_1\beta_1$ complexes are found in the detergent extracts (Fig. 4*e*). Thus, mutation of Asp^{253} to an Ala leads to a loss of ligand binding capacity.

Cell Adhesion Properties of Mutated α_1 Subunits—In addition to affinity chromatography experiments, we performed cell adhesion experiments with HA1, cID, and PM848 cells. When plated on with Col IV, both HA1 and cID cells (Fig. 5*A*) exhibit cell adhesion to low concentrations of the Col IV. PM848 (Fig. 5*A*) and parental 3T3 cells (not shown; see Briesewitz *et al.* (33)) do not bind to this substrate even at high concentrations. Adhesion to LN, another known ligand for $\alpha_1\beta_1$, shows a similar result; HA1 and cID bind to low concentrations, whereas PM848 cells need high concentrations for adhesion.

Inhibition studies (Fig. 5*B*) with the functional mAb 1B3.1 show results which parallel the immunoprecipitation analysis shown above. Only HA1 adhesion to Col IV or LN is inhibited by 1B3.1, cID cells are not inhibited, since 1B3.1 does not recognize the chimeric α subunit. The adhesion of PM848 cells to high concentrations of LN is not inhibited by the functional mAb (not shown).

Finally, we tried to rescue the loss of ligand binding capacity of the mutant $\alpha_1\beta_1$ dimer by using high concentrations of Mn^{2+} ions in the cell adhesion media. PM848 cells did not differ from the parental 3T3 cells when plated on Col IV (not shown). Both cell lines showed a minor increase in adhesion as compared to the adhesion observed in the presence of Mg^{2+} ions. Since this

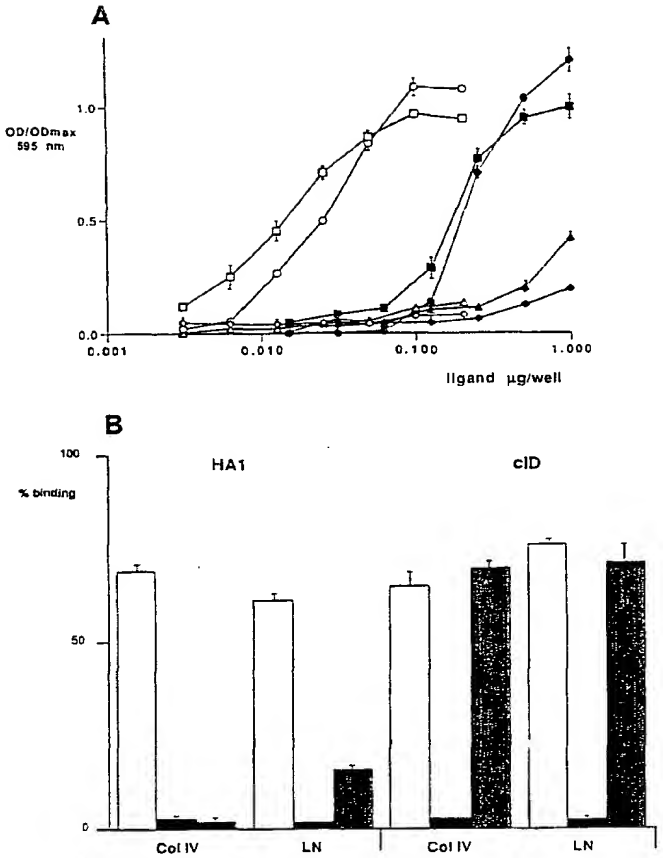

FIG. 5. **Cell adhesion assay of NIH 3T3 cell lines.** *A*, HA1 (*squares*), cID (*circles*), PM848 (*triangles*) or nontransfected NIH 3T3 (*diamonds*) cells were assayed for adhesion to Col IV (open symbols) or LN (*closed symbols*). Amounts of adhesive proteins indicated at the *bottom* of the graphs were dried down on microtiter plates. Cells were incubated for 30 min at 37 °C and washed, and the bound cells were fixed and stained. Optical density was read at 595 nm and compared to cells bound to an excess of FN (1 µg/well). HA1 and cID cells have similar adhesion curves on Col IV and LN. NIH 3T3 and PM848 cells do not bind to Col IV and bind only to high concentrations of LN. Inhibition studies are shown in *B*. Microtiter wells were coated with Col IV (0.05 µg/well, HA1; 0.08 µg/well, cID) or LN (0.25 µg/well, HA1; 0.5 µg/well for cID). Cells were incubated for 30 min at 37 °C in the presence of 5 mM EDTA (*black bars*), anti-human α_1 mAb1B3.1 (1:200, *gray bars*), or in the absence of inhibitor (*open bars*). Cell binding was determined as mentioned above. Both cell lines bind to Col IV and LN in a divalent cation-dependent manner. mAb 1B3.1 blocks HA1 cell adhesion, but not cID adhesion.

effect was also observed on parental 3T3 cells, we conclude that it is related to a Mn^{2+} influence on mouse α subunits and not on the presence of the heterologous α_1 (PM848) subunit.

DISCUSSION

The experiments in this study investigate the domains within the α_1 subunit that contribute to ligand binding. This integrin is a known receptor for collagens and LN(s). We do not exclude that the β1 subunit is also involved in ligand recognition, and data from several groups have shown that β domains are required for binding (12, 13, 36), but our present work is focused on the role of α subunits in the specificity of ligand recognition. The α subunits clearly are involved in site specificity since a change in α composition of an integrin can lead to binding to a different site within the ligand. For example, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ interact with different domains within the cell binding fragment of collagen type IV (35), and the binding sites within LN for $\alpha_1\beta_1$ and $\alpha_6\beta_1$ are in distinct domains (37).

We began this study by mapping domains required for the binding of the function blocking anti-α_1 monoclonal antibody 1B3.1. This antibody effectively blocks binding of $\alpha_1\beta_1$ to both Col IV and LN. We isolated from chicken mRNA a partial cDNA which includes the entire I domain of avian α_1, and exchanged it for the human I domain within full length human α_1. This chimeric protein shows the same functions as the wild type protein, in that it dimerizes with the mouse β_1 subunit when expressed in NIH 3T3 mouse fibroblasts, it enables the cells to adhere and spread on Col IV and LN, and the isolated dimer binds to its ligand in affinity chromatography experiments. However, 1B3.1 does not bind to the chicken/human α_1 protein. Therefore, binding of 1B3.1 to $\alpha_1\beta_1$ requires the presence of the human I domain. A similar result was reported for the leukocyte integrin α_X (p150/90 α subunit) (18). By swapping the p150 and the Mac-1 I domains, the authors located most of the α_X blocking monoclonal antibodies into this part of the protein. In addition, Landis *et al.* (38) mapped the epitope of an activating anti-human LFA-α monoclonal antibody to the I domain. Clearly, this inserted domain is critical for receptor function.

A number of experiments strongly suggest that the I domain is involved in ligand recognition and cation binding. There are five α subunits, α_1, α_2, α_{LFA-1}, α_{Mac-1}, and α_{p150}, which have I domains. There are several features of this group of α subunits that are unique. First, is the preference for Mg^{2+}, for which Ca^{2+} cannot substitute (39, 40). Indeed, Grzesiak *et al.* (41) have shown that high levels of Ca^{2+} can inhibit the function of $\alpha_2\beta_1$. Very recently, the role of the I domain in binding divalent cations has been demonstrated. Expression of this segment as a bacterial fusion protein led to the recovery of a protein fragment capable of binding several divalent cations (17). Interestingly, this region preferred Mn^{2+}, then Mg^{2+} with Ca^{2+} bound less well. This order of preference exactly parallels that of all of the I domain containing integrins. Furthermore, mutation of an aspartate residue within the I domain of full length Mac-1 led to a receptor with impaired ligand binding properties. Accordingly, we mutagenized the homologous oxygenated residue within the human α_1 I domain, converting an aspartate residue into an alanine residue. By expressing this mutant form of α_1 in 3T3 cells we were able to demonstrate that these cells, in contrast to HA1 cells expressing the wild type human α_1, do not adhere to Col IV and that the isolated receptor does not bind to Sepharose-bound CB3 fragment. The epitope recognized by the functional mouse monoclonal antibody 1B3.1 was not affected by this mutation. Thus, a similar mechanism of ligand recognition is present in both α_1 and α_M, although they recognize different ligands. Our data suggests that the I domain is central for ligand binding in the five α subunits which have this domain. In addition, Kamata *et al.* (42) have recently demonstrated similar findings with the integrin $\alpha_2\beta_1$.

What about the role of other cation binding domains in $\alpha_1\beta_1$ ligand binding? We have attempted to address this question in several ways. We have used peptide competition experiments to test the contribution of these regions in ligand binding. None of these peptides nor a peptide derived from the I domain containing Asp^{253} demonstrated a specific competition even at concentrations of 2–4 mM (not shown). It is questionable whether the conformation of short peptides is similar to the mature protein and thus the lack of inhibition does not rule out an involvement of the metal binding domains. Thus the exact role of these cation binding regions remains unclear. Interestingly, PM848 cells and HA1 cells blocked with mAb 1B3.1 (not shown) bind slightly better to laminin than the parental NIH 3T3 cells, which suggests a role of non-I domain regions. We have also attempted to make site directed mutants of the metal binding

domains, changing critical aspartate residues to glutamate residues within repeat five. However, all of these constructs were retained intracellularly when expressed in cells.[3] Thus it seems likely that these metal binding domains are required for proper structure of the I domain containing α subunits, while the I domain may be the primary determinant of ligand recognition. The point mutation within the I domain does not interfere with surface expression of an $\alpha_1\beta_1$ heterodimer recognized by mAb 1B3.1. We conclude that the overall structure of the α_1 subunit is not strongly affected. We are currently trying to address this question by using I domain swaps to determine whether this domain can confer a specific site recognition within a ligand.

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Integrin α2 I-domain is a binding site for collagens

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SUMMARY

Integrins α1β1 and α2β1 are major cellular receptors for collagens. The α1 and α2 subunits contain a ~200 amino acid inserted domain (I-domain) in their N-terminal region and, because of the homology between the I-domains and the collagen-binding A-domains of von Willebrand factor, it has been suggested that the I-domains might mediate the collagen-binding functions of α1β1 and α2β1. In order to fully investigate this hypothesis, we have generated recombinant human α2 I-domain (rα2I) by reverse transcriptase-polymerase chain reaction/bacterial expression and tested its ability to mediate the collagen-binding functions of α2β1. Rα2I binds specifically to type I collagen in a concentration-dependent manner: binding is cation dependent and, like the complete receptor, is supported by magnesium and manganese ions but not by calcium ions. Rα2I is recognised by anti-functional anti-α2 monoclonal antibodies 6F1, 5E8 and P1E6 in capture ELISAs, and anti-functional antibodies inhibited rα2I-collagen binding. In addition, rα2I inhibits cell spreading on collagen. Rα2I is therefore a collagen-binding domain and can account for many of the collagen-binding functions of integrin α2β1. We have also determined the collagen specificity of rα2I and found that it binds types I, II and XI collagen.

Key words: collagen, integrin, I-domain, A-domain

INTRODUCTION

The integrins are a family of αβ heterodimeric receptors which mediate cell-cell and cell-extracellular matrix interactions (Hynes, 1992; Tuckwell and Humphries, 1993). Collagens are important integrin ligands and two integrins, α1β1 and α2β1, are recognised as collagen receptors (Wayner and Carter, 1987; Takada et al., 1988; Kramer and Marks, 1989). The α1 and α2 subunits, along with the αL, αM, αX and αE (αIEL) subunits are distinguished from other α subunits by the presence of a ~200 amino acid inserted domain (I-domain) in their N-terminal region (Hughes, 1992; Shaw et al., 1994). This I-domain is homologous to the von Willebrand factor A-domains, and A-domain-like sequences are also found in a number of other proteins including type VI collagen, complement factor B and cartilage matrix protein (reviewed by Colombatti et al., 1993). Since the A-domains of von Willebrand factor have been shown to bind collagens (Roth et al., 1986; Pareti et al., 1987; Mohri et al., 1989; Piétu et al., 1989), it has been proposed that the collagen-binding functions of integrins α1β1 and α2β1 might be mediated by their I-domains (Pytela, 1988). In support of this, a number of anti-functional anti-α2 antibodies have recently been shown to map to the I-domain (Kamata et al., 1994).

In order to fully investigate the role of the I-domain, we have generated recombinant human α2 I-domain (rα2I) by reverse transcriptase-polymerase chain reaction/bacterial expression and tested its ability to mediate the collagen-binding functions associated with α2β1. In common with the integrin, rα2I binding to collagen is specific, concentration-dependent and requires manganese or magnesium ions. Anti-functional anti-α2 antibodies recognised rα2I and inhibited rα2I-collagen binding. In addition, rα2I specifically inhibited cell spreading on collagen. Rα2I interacted specifically with types I, II and XI collagen.

MATERIALS AND METHODS

Oligonucleotides were synthesised on an Applied Biosystems 391 DNA synthesiser. Antibodies were obtained from the following sources: anti-integrin α2 mouse monoclonal antibodies; P1E6 (Carter et al., 1990), Gibco, Paisley, Scotland, UK; 6F1 (Santoro et al., 1988), V. Woods, University of California, San Diego, CA, USA; 12F1 (Coller et al., 1989), B. Coller, State University of New York, Stony Brook, NY, USA; 5E8 (Chen et al., 1991), R. Bankert, Roswell Park Cancer Institute, Buffalo, NY, USA; 1C11 was raised as described by Mould et al. (1991); polyclonal anti-integrin α2β1 antiserum 999 (Kern et al., 1993), A. Kern and K. Kühn, Max-Planck-Institut für Biochemie, Martinsried, Germany; anti-integrin αv mouse monoclonal LM142, D. Cheresh, Scripps Clinic, La Jolla, CA, USA; anti-integrin β1 rat monoclonal mAb13, K. Yamada and S. Akiyama, NIDR, NIH, Bethesda, MD, USA.

Rat tail type I collagen and human placental types IV and V collagen were obtained from Sigma, Poole, Dorset, UK. Human placental types I and VI collagen were obtained from Lab Impex, Teddington, Middlesex, UK. Bovine types II, IX and XI collagen were purified from articular cartillage as described by Ayad et al. (1981). Types II and XI collagen were at least 95% pure by SDS-PAGE while type IX collagen contained low levels of type XI collagen as its only visible contaminant.

Generation of recombinant integrin α2 I-domain (rα2I)

A cDNA clone corresponding to the integrin α2 I-domain was generated by reverse transcriptase-polymerase chain reaction. RNA was prepared from HT1080 human fibrosarcoma cells as described by Makarem et al. (1994) and first strand cDNA was generated from this using a 3′ primer spanning the predicted intron boundary site at the 3′ end of the I-domain and incorporating an *Eco*RI site (5′-GGGAATTCAACAGTACCTTCAATGCTG-3′). Polymerase chain reaction (PCR) amplification of this cDNA was then carried out using the above 3′ primer and a 5′ primer designed to give a 5′ extension corresponding to the integrin sequence preceding the I-domain (Michishita et al., 1993), and incorporating a *Bam*HI site (5′-GGG-GATCCAGTCCTGATTTTCAGCTCTCAG-3′). 50 cycles were carried out, each cycle consisted of 1 minute at 94°C, 1 minute at 55°C and 2.5 minutes at 72°C. PCR products were run on a 1% agarose gel, the major band (which was of the correct M_r) excised, digested with *Bam*HI and *Eco*RI, ligated into pUC119, and used to transform *E. coli* strain DH5αF′. The rα2I sequence from transformants was sequenced by the dideoxy chain termination method of Sanger et al. (1977) and compared with the published sequence (Takada and Hemler, 1989). Rα2I cDNA was then subcloned into pGEX-2T (Smith and Johnson, 1988; Pharmacia, Milton Keynes, UK) and used to transform DH5αF′. Transformants were screened by using 0.5 ml of overnight cultures of transformants to innoculate 5 ml of LB, 50 mg/ml ampicillin. Cultures were grown for 1 hour at 37°C and then for 4 hours at 37°C in the presence of 0.1 mM isopropyl-β-D-thiogalactoside (IPTG). Cultures were then centrifuged, bacterial pellets solubilised in SDS-PAGE sample buffer and resolved on 10% acrylamide SDS-PAGE gels. Transformants expressing the glutathione *S*-transferase-rα2I fusion protein were identified by the presence of a protein band of ~50 kDa.

For the purification of rα2I, typically a 40 ml overnight culture of transformants was used to innoculate 400 ml of LB, 50 mg/ml ampicillin. The culture was grown for 1 hour at 37°C and then induced for 4 hours with IPTG as above. Cells were harvested by centrifugation (4,500 *g*, 10 minutes) and pellets resuspended in PBS without divalent cations (PBS^-). Suspensions were then sonicated, centrifuged (2,500 *g*, 10 minutes) and the supernatants retained. Pellets were resuspended in PBS^-, sonicated and centrifuged a further 2 times and the supernatants pooled. The resulting lysate was then passed down a glutathione agarose column (Sigma; prepared according to manufacturers instructions) equilibrated in 150 mM NaCl, 20 mM Tris-HCl, pH 7.5 (TBS), the column washed with 10 volumes of TBS and the glutathione S-transferase-rα2I fusion protein eluted with 5 mM glutathione in 50 mM Tris-HCl, pH 8.0. The fusion protein was then dialysed against TBS to remove the glutathione and cleaved with thrombin (Sigma; 1:100 (w/w) enzyme:fusion protein) in the presence of 2.5 mM $CaCl_2$ for 3 hours at room temperature. The cleavage mixture was reduced by the addition of dithiothreitol (DTT) to 5 mM to break a disulphide bond between the glutathione *S*-transferase and rα2I moieties (D. S. Tuckwell, unpublished observation) and then passed a second time down the glutathione agarose column to remove glutathione *S*-transferase. The rα2I remained in the flowthrough and was dialysed to remove DTT. For experiments requiring high concentrations of rα2I, samples were concentrated using a Microcon 3 microconcentrator (Amicon, Stonehouse, Gloucester, UK). The recombinant I-domain produced was 224 amino acids long, starting GSS(124)PDFQ...IEGTV(339)**EFIVTD**; bold letters indicate non-integrin amino acids, numbers correspond to the integrin sequence.

Biotinylation of rα2I was carried out as follows: Rα2I was dialysed into PBS^- and 1/5 volume 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.0, added to elevate the pH for biotinylation. Sulpho-NHS-biotin (Pierce, Chester, UK) was added at 1:2 (w/w) rα2I:biotin and incubated for 1 hour at room temperature. The mixture was then dialysed against TBS to remove unincorporated biotin.

Rα2I solid-phase binding assays

The rα2I binding assay was based on the solid phase assay of Makarem et al. (1994) and the cell attachment assay (Tuckwell et al., 1994). Microtitre plates (Costar, Cambridge, MA, USA) were coated with ligand molecules, dissolved in PBS^-, for 1 hour at room temperature. Wells were then blocked with 50 mg/ml BSA (Sigma) in TBS for 1 hour at room temperature, washed twice with TBS, 1 mM $MgCl_2$, and biotinylated rα2I added at a concentration of 0.25 mg/ml in TBS, 1 mM $MgCl_2$, 1 mg/ml BSA (Calbiochem, Beeston, Nottingham, UK), for 3 hours at 35°C. Wells were then washed three times with TBS, 1 mM $MgCl_2$, and extravidin peroxidase (Sigma) added at a concentration of 10 μg/ml in TBS, 1 mM $MgCl_2$, 1 mg/ml BSA (Calbiochem) for 10-20 minutes at room temperature. Wells were washed three times with TBS, 1 mM $MgCl_2$, and bound rα2I visualised with 0.1 M sodium acetate, 0.05 M NaH_2PO_4, 2 mM 2′2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 0.03% H_2O_2. Wells were read at 405 nm on a plate reader. To enable comparisons between assays, data from different assays were normalised, typically to rα2I binding to 100 mg/ml type I collagen in the presence of 1 mM $MgCl_2$.

For assays where agents were to be added exogenously or cation concentrations varied, rα2I and agents were made up to double the final concentration and half of final volumes added of each. Agents (antibodies, divalent cations) were always added before rα2I. In addition, for assays studying cation dependencies, the wash after blocking was carried out with TBS without cations, and subsequent washes and incubations included 1 mM $MnCl_2$ in addition to 1 mM $MgCl_2$.

Solid phase assays which employed antibody detection of bound rα2I, rather than extravidin peroxidase, were carried out as follows: The above protocol was used up to and including the three washes following incubation with I-domain. To each well was then added polyclonal anti-α2β1 antiserum 999, 1:400 in TBS, 1 mM $MgCl_2$, 1 mg/ml BSA, and plates were incubated for 45 minutes at room temperature. Wells were then washed three times with TBS, 1 mM $MgCl_2$, and peroxidase-linked goat anti-rabbit antibody (Sigma) added, 1:800 in TBS, 1 mM $MgCl_2$, 1 mg/ml BSA, followed by a further 45 minute incubation. Wells were then washed three times with TBS, 1 mM $MgCl_2$ and detected with ABTS as above.

Rα2I inverted binding assay

This assay was based on the solid phase binding assay, but with the soluble and immobilised phases reversed. Type I collagen was biotinylated by the addition of sulpho-NHS-biotin (1:1 (w/w) collagen:biotin) to collagen solutions in 1:1.5 (v/v) 0.1 M acetic acid: PBS^- and incubated for 1 hour at room temperature. The collagen solution was then dialysed against 0.1 M acetic acid to remove unincorporated biotin. Assays were carried out as follows: microtitre plates were coated with rα2I at 25 μg/ml in PBS^- for 1 hour at room temperature. Wells were then blocked with 50 mg/ml BSA in TBS for 1 hour at room temperature, washed twice with TBS, 1 mM $MnCl_2$, and biotinylated type I collagen in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $MnCl_2$, 1 mg/ml BSA added. Plates were incubated for 3 hours at 35°C. Wells were then washed three times with TBS, 1 mM $MnCl_2$, and bound collagen visualised with extravidin peroxidase as for solid-phase binding assays.

Capture ELISA

Capture ELISAs were carried out after the method of Brookman et al. (1995). Immulon 4 microtitre plates (Dynatech, Chantilly, VA, USA) were coated with antibodies at a concentration of 10 μg/ml (purified antibodies) or 1:100 ((v/v) ascites) in 50 mM Na_2CO_3, pH 9.5, overnight at 4°C. Wells were washed twice with PBS^-, blocked with PBS^-, 2% (w/v) fat-free milk powder for 1 hour at 4°C, washed twice with PBS^-, 0.1% Tween-20, and incubated with 10 μg/ml biotinylated rα2I in PBS^-, 0.1% Tween-20, 0.5% (w/v) fat-free milk powder for

2 hours at 4°C. Wells were then washed three times with PBS⁻, 0.1% Tween-20, incubated with extravidin peroxidase (Sigma) at a concentration of 4 μg/ml in PBS⁻, 0.1% Tween-20, 0.5% (w/v) fat-free milk powder for 2 hours at 4°C, washed three times with PBS⁻, 0.1% Tween-20, and bound antibody visualised with ABTS as above.

Cell spreading assays

HT1080 human fibrosarcoma cells were maintained as described by Weston et al. (1994). Microtitre plates (Costar) were coated with human type I collagen at 2.5 μg/ml in PBS⁻ or 80 kDa fragment of fibronectin (prepared as described by Garcia-Pardo et al., 1989) at 5 μg/ml in PBS⁻ for 1 hour at room temperature. These concentrations were found to be the lowest concentrations which promoted maximal spreading. Wells were then blocked with heat denatured BSA (Sigma; Humphries et al., 1986) for 1 hour at room temperature. Cells were removed from culture flasks with trypsin/EDTA, trypsin was quenched with culture medium, the cell suspension washed twice with MEM and cells resuspended to 5×10^5 cells/ml in MEM. 80 μl of rα2I diluted in phosphate buffered saline with divalent cations was added to each well of the microwell plate, followed by 20 μl of cell suspension. Plates were incubated under normal culture conditions for 1. Wells were then fixed by direct addition of 10 ml 50% (w/v) glutaraldehyde and the number of spread cells (phase-dark cells with a flattened or flattening morphology) recorded.

RESULTS

Integrin α2 I-domain cDNA was generated from HT1080 human fibrosarcoma RNA by reverse transcriptase-polymerase chain reaction. α2 I-domain cDNA was then sequenced, ligated into the pGEX-2T vector and used to transform *E. coli*. Transformants synthesised a glutathione *S*-transferase-recombinant integrin α2 I-domain (rα2I) fusion protein of ~50 kDa after IPTG induction (Fig. 1). The fusion protein was purified on a glutathione-agarose column and, after thrombin cleavage and removal of the glutathione *S*-transferase moiety by a second round of glutathione-agarose affinity chromatography, yielded a protein of ~25 kDa, the expected size for the 224 amino acid rα2I. The recombinant protein was at least 90% pure by SDS-PAGE (Fig. 1).

In order to investigate the functional role of the integrin α2 I-domain, experiments were carried out to demonstrate the extent to which rα2I accounts for the collagen-binding function of integrin α2β1. In solid-phase ligand-binding assays using soluble biotinylated I-domain, rα2I bound to the α2β1 ligand, type I collagen (Fig. 2). Heat denaturation of type I collagen, which is known to dramatically reduce its ability to support α2β1 binding (e.g. Tuckwell et al., 1994), abolished rα2I binding. Rα2I was also unable to bind to the 80 kDa fragment of fibronectin or to fibrinogen, neither of which are either collagenous proteins or ligands for α2β1 although both are adhesive proteins. In addition, pre-treatment of collagen with bacterial collagenase reduced the amount of rα2I binding to background levels (data not shown): rα2I binding to type I collagen was therefore specific. The binding of rα2I to type I collagen was concentration-dependent and saturable (Fig. 3), reinforcing the demonstration of the specificity of the interaction. No binding to the 80 kDa fragment of fibronectin was seen over the same concentration range. Rα2I binding to collagen could also be demonstrated in an inverted binding assay, resembling the solid-phase binding assay but with immobilised rα2I and biotinylated type I collagen in solution:

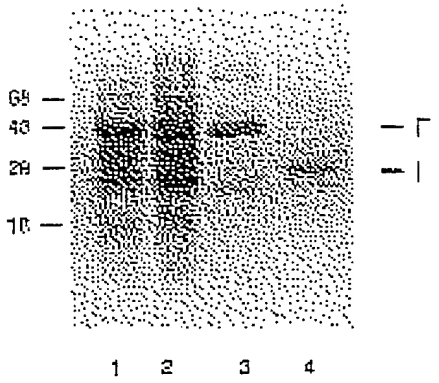

Fig. 1. SDS-PAGE gel demonstrating the purification of rα2I: *E. coli* transformed with pGEX-2T carrying rα2I cDNA were induced with IPTG. Lane 1, induced bacteria, solubilised in SDS-PAGE sample buffer; lane 2, PBS⁻ lysate of induced bacteria; lane 3, glutathione *S*-transferase-rα2I fusion protein retained on glutathione-agarose column; lane 4, rα2I product after cleavage of fusion protein and removal of glutathione *S*-transferase moiety. F marks the position of the glutathione *S*-transferase-rα2I fusion protein, I marks the position of rα2I.

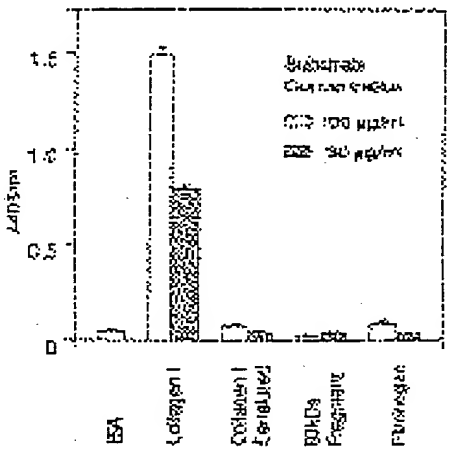

Fig. 2. Binding of biotinylated rα2I to different substrates in a solid phase assay, two substrate concentrations are shown; mean ± s.e.m., n=10 from 2 experiments. Solid phase assays were carried out essentially as follows: microtitre plates were coated with substrate molecules, blocked with BSA and biotinylated rα2I added at 0.25 μg/ml. Unbound rα2I was then washed off, extravidin-peroxidase added, unbound peroxidase washed off and the colourimetric peroxidase substrate ABTS added. Plated were read on a plate reader at 405 nm. Type I collagen was heat denatured by heating solutions to 50°C for 30 minutes immediately prior to coating microtitre wells; 80 kDa fragment, 80 kDa fragment of fibronectin.

type I collagen bound to rα2I, but binding was greatly reduced by prior heat denaturation of the collagen (Fig. 4).

The interaction of integrin α2β1 with type I collagen is dependent on divalent cations (Santoro, 1986; Grzesiak et al., 1992). Consistent with this, rα2I binding to type I collagen was greatly reduced by the addition of 10 mM EDTA (Fig. 5). A more detailed study of the divalent cation dependency of rα2I

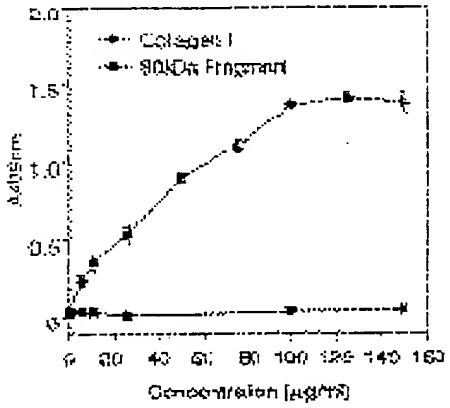

Fig. 3. Concentration-dependence of biotinylated rα2I binding to type I collagen and 80 kDa fragment of fibronectin over a range of substrate concentrations in a solid phase assay; mean ± s.e.m., n=8 from 2 experiments.

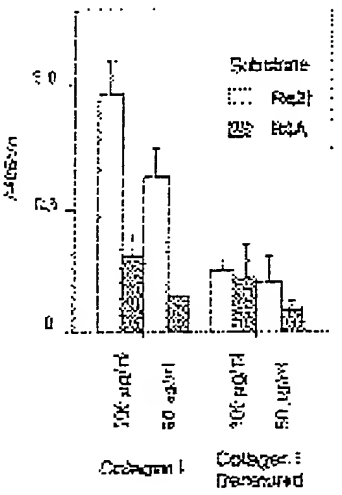

Fig. 4. Binding of biotinylated type I collagen to rα2I in an inverted binding assay. Microtitre plates were coated with rα2I, blocked with BSA and biotinylated collagen added. Unbound collagen was then washed off, extravidin-peroxidase added, unbound peroxidase washed off and the colourimetric peroxidase substrate ABTS added. Plated were read on a plate reader at 405 nm. Denatured collagen was generated by heating solutions to 50°C for 30 minutes prior to addition to microtitre wells. Collagen was added at either 100 μg/ml or 50 μg/ml; for controls, binding of biotinylated collagen to BSA was also measured.

binding to type I collagen showed that magnesium and manganese ions supported binding, with manganese ions supporting a greater level of binding than magnesium ions, while calcium ions were inactive (Fig. 6). These data are consistent with the observed cation dependency of integrin α2β1 where magnesium ions support binding but calcium ions only support a low level of binding or are inhibitory (Santoro, 1986; Grzesiak et al., 1992; Kern et al., 1993). The enhancement of binding seen with manganese has also been observed for other

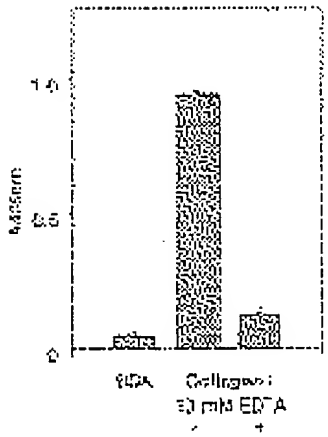

Fig. 5. Inhibition of biotinylated rα2I binding to type I collagen by 10 mM EDTA in a solid phase assay; mean ± s.e.m., n=8 from 2 experiments. Microtitre wells were coated with 100 μg/ml type I collagen.

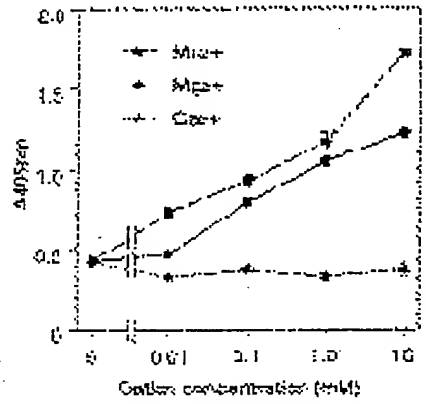

Fig. 6. Divalent cation dependency of rα2I binding to type I collagen in a solid phase assay; mean ± s.e.m., n=12 from 2 experiments. Microtitre wells were coated with 30 μg/ml type I collagen, corresponding approximately to the half-maximal level of binding (see Fig. 3). This enables optimal promotion or inhibition of binding by cations to be observed.

integrin ligands (Gailit and Ruoslahti, 1988; Kern et al., 1993). Divalent cation dependency of binding was also studied over a range of I-domain concentrations: a divalent cation-independent component of binding was observed as I-domain concentration was increased (Fig. 7A), and this could be inhibited by the anti-functional antibody 5E8 (data not shown). Cation dependency of the binding of unmodified I-domain, detected with a polyclonal antibody, was also studied (Fig. 7B). However, binding was seen to be completely divalent cation-dependent.

A number of anti-integrin α2 monoclonal antibodies have been described which inhibit α2β1 function; these antibodies were therefore used in capture ELISA assays to test their ability to recognise rα2I. The antibodies 6F1 (Coller et al., 1989), 5E8 (Chen et al., 1991) and P1E6 (Carter et al., 1990)

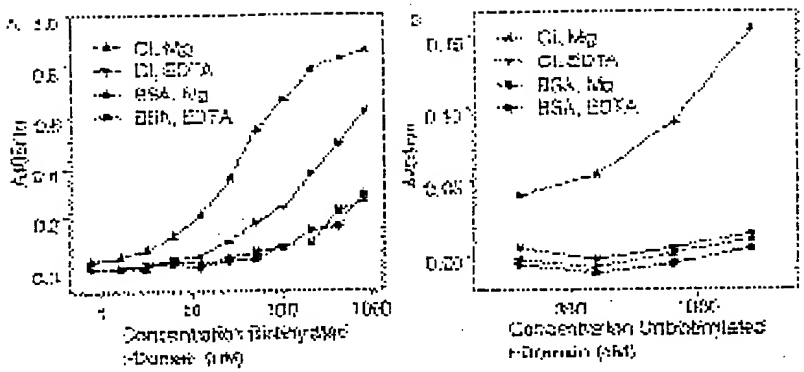

Fig. 7. Divalent cation dependency of I-domain binding to collagen over a range of I-domain concentrations. (A) Representative experiment showing binding of biotinylated rα2I to type I collagen (coated at 150 μg/ml). (B) Representative experiment showing binding of unbiotinylated rα2I to type I collagen (coated at 100 μg/ml), detected with polyclonal antiserum. 1,000 nM I-domain is equivalent to 25 μg/ml.

all bound rα2I (Fig. 8A) demonstrating that their anti-functional effect is mediated through binding to rα2I and supporting the proposed role of rα2I in integrin-collagen binding. The anti-α2 antibody 12F1 (Santoro et al., 1988) also bound rα2I, although this antibody is not anti-functional. A control anti-α2 antibody 1C11 (Mould et al., 1991) and a number of irrelevant antibodies did not bind rα2I. Preliminary data also showed that a number of other anti-α2 antibodies, 10A4 and 31H4 (Mould et al., 1991), and HAS3, HAS4 and the anti-functional antibody HAS6 (Tenchini et al., 1993), did not bind rα2I in the capture ELISA. The effect of the anti-functional antibody 5E8 on rα2I-collagen binding was also studied: 5E8 inhibited I-domain binding whereas the control antibody 1C11 had no effect (Fig. 8B). 6F1 also inhibited I-domain binding to collagen (data not shown).

In order to further demonstrate the role of the integrin α2 I-domain as a collagen-binding site, rα2I was tested for its ability to inhibit HT1080 spreading on collagen, a process mediated by integrin α2β1 (Wayner and Carter, 1987; Weston et al., 1994). Rα2I was seen to inhibit spreading of HT1080 cells on type I collagen but not on the 80 kDa fragment of fibronectin (Fig. 9A,B). Inhibition of HT1080 spreading on type I collagen plateaued at 30-40% in the presence of 2-4 mg/ml rα2I (Fig. 9C). However, it was apparent that even those cells which were spread on collagen in the presence of rα2I were less well spread than cells spread on 80 kDa fragment of fibronectin (Fig. 9A,B).

Finally, the specificity of rα2I-collagen binding was examined using a range of collagens as substrates in solid-phase ligand-binding assays (Fig. 10). Types I and II collagen supported rα2I binding well, as did type XI collagen. In contrast, little or no binding was observed to heat-denatured type II collagen or to types IV, V, VI or IX collagen. The inability of these latter collagens to support binding was not due to differences in coating of the microtitre dishes as heat-denatured type II collagen coats with the same efficiency as native type II collagen (Tuckwell et al., 1994), and type IV, V, VI, and IX collagens all supported at least 50% spreading of HT1080 cells (data not shown). The use of anti-functional anti-α2 monoclonal antibodies also showed that HT1080 cell spreading on type IV collagen was α2β1-dependent.

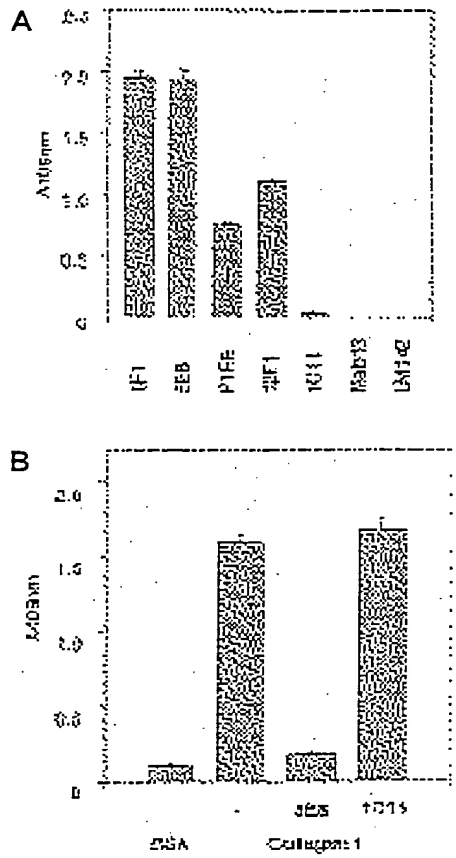

Fig. 8. The interactions of anti-functional anti-α2 antibodies with rα2I. (A) Capture ELISA using biotinylated rα2I and anti-α2 antibodies. Microtitre plates were coated with antibodies, blocked, and biotinylated rα2I added. Unbound antibody was washed off, extravidin-peroxidase added, unbound peroxidase was washed off and the colourimetric peroxidase substrate ABTS added. Plated were read on a plate reader at 405 nm. 6F1, 5E8, P1E6, antifunctional mouse anti-α2 monoclonal antibodies; 12F1, 1C11 other mouse anti-α2 monoclonal antibodies; mAb13, rat anti-integrin β1 monoclonal antibody; LM142 mouse anti-integrin αv monoclonal antibody. P1E6 was supplied as ascites as was LM142, used here as an ascites control. (B) Inhibition of binding of biotinylated rα2I to type I collagen by the antifunctional antibody 5E8 in solid phase assays; mean ± s.e.m, n=6 from 2 experiments. Microtitre plates were coated with 100 μg/ml type I collagen; 5E8 and control antibody 1C11 were added at 10 μg/ml final concentration.

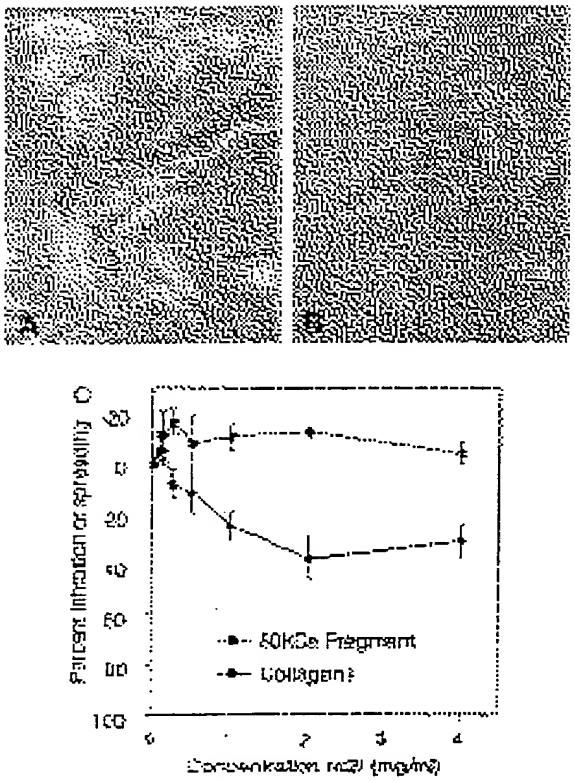

Fig. 9. Inhibition of HT1080 spreading on type I collagen by rα2I. Microwell plates were coated with type I collagen or 80 kDa fibronectin fragment and blocked with heat denatured BSA. I-domain solution was added, followed by HT1080 cell suspension. Plates were incubated for 1 hour at 37°C and fixed by direct addition of glutaraldehyde. (A,B) HT1080 spreading on type I collagen (A) or 80 kDa fragment of fibronectin (B) in the presence of 2 mg/ml rα2I. Note that most cells in (A) are phase bright with a small surface area compared to cells in (B). (C) The percentage inhibition of HT1080 spreading on type I collagen or 80 kDa fragment of fibronectin in the presence of increasing concentrations of rα2I: representative experiment shown, mean ± s.d., n=4.

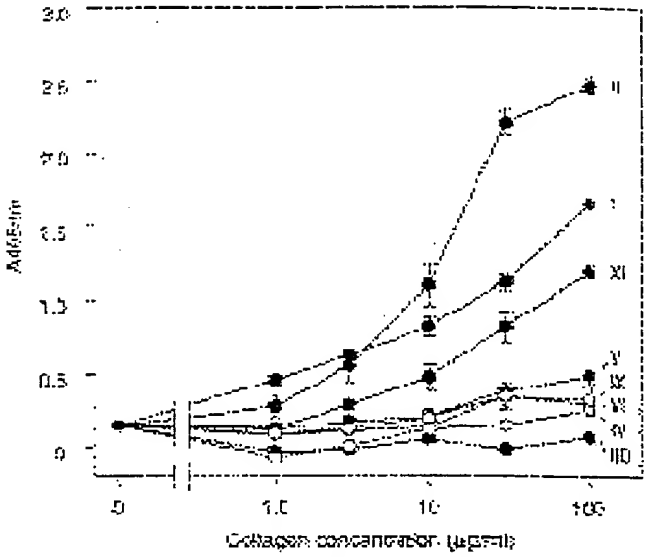

Fig. 10. Binding of biotinylated rα2I to a range of collagens in a solid phase assay; IID, denatured type II collagen; mean ± s.d., n=8 from 2 experiments.

DISCUSSION

We report the generation and testing of a recombinant human integrin α2 I-domain (rα2I). In common with integrin α2β1, the interaction of rα2I with type I collagen was seen to be specific and concentration-dependent. The rα2I-type I collagen interaction required divalent cations, and was supported by manganese and magnesium ions but not by calcium ions. Rα2I was also the binding site for a number of anti-functional anti-α2 antibodies and anti-functional antibodies inhibited rα2I-collagen binding. Rα2I also specifically inhibited cell spreading on type I collagen. Rα2I is therefore a collagen-binding domain within α2β1 and is able to account for many of the collagen-binding properties of the integrin.

Integrin α2β1-collagen binding

Integrins α1β1 and α2β1 are recognised as receptors for collagens (Wayner and Carter, 1987; Takada et al., 1988; Kramer and Marks, 1989). The integrin α subunits can be grouped on the basis of certain primary sequence characteristics, reflecting the underlying degree of sequence identity (Hughes, 1992; Hynes, 1992). α1, α2, αL, αM, αX and αE (αIEL) have in common an inserted domain in their N-termini known as the I-domain, although within this group α1 and α2 are more similar to each other than to the other α subunits. The I-domain is homologous to the von Willebrand factor (vWf) A-domains (reviewed by Colombatti et al., 1993), and since vWf A-domains have been shown to bind collagen (Roth et al., 1986; Pareti et al., 1987; Mohri et al., 1989; Piétu et al., 1989), it has been hypothesised that the collagen-binding functions of integrins α1β1 and α2β1 might be provided by the I-domains (Pytela, 1988). Recently, Kamata et al. (1994) showed that a number of anti-functional anti-α2 antibodies mapped to the I-domain, and Kern et al. (1994) have made a similar observation for α1, thus providing some support for this hypothesis. The data reported here for rα2I conclusively demonstrates that the I-domain of α2 is a collagen-binding domain.

Rα2I bound types I, II and XI collagen, and preliminary data indicated that rα2I also bound type III collagen. The fibril-forming collagens, types I, II, III, V and XI, are grouped together on the basis of molecular and physical characteristics, with a further subgrouping into types I, II and III, and types V and XI (Kielty et al., 1993). On the basis of this, the binding of rα2I to types I and II can be rationalised. The inability of type V to support binding while type XI was active may be due to the marked similarity, or perhaps identity, of α3(XI) and α1(II) (Burgeson and Hollister, 1979). Types IV, VI and IX collagen do not closely resemble the fibril-forming collagens (Kielty et al., 1993) and their different sequences and structures is probably the basis of their different behaviour in these assays compared to the fibrillar collagens.

Although binding of rα2I to collagens IV, V, VI or IX could not be detected, α2β1 has been clearly shown to act as a receptor for type IV collagen (e.g. Wayner and Carter, 1987; Kern et al., 1993). In our hands, HT1080 cells, which express α2β1 as the sole collagen receptor, adhere better to type I collagen than to type IV collagen (D. S. Tuckwell and M. J. Humphries, unpublished work) and it is therefore possible that rα2I binding to type IV collagen was too weak to be detected. Furthermore, the molecular basis of collagen recognition by integrins is still far from clear, and it is not known to what extent the fibrillar collagens and type IV collagen share a common integrin recognition motif.

While rα2I clearly binds certain fibrillar collagens, it is not certain that the I-domain is solely responsible for integrin-collagen binding. Comparisons with vWf, where A-domains bind collagen, would suggest that the integrin I-domain can act as the sole binding site on the integrin for collagen. Alternatively, the I-domain could function as a facilitating module, binding collagens with a relatively low affinity but enabling recognition of a binding sequence by other parts of the integrin molecule; This might account for the inability of rα2I to completely inhibit cell spreading on type I collagen (see Fig. 9). Alternatively, assay conditions might not have enabled sufficiently high amounts of correctly folded protein to be present before secondary effects took over, e.g., even at the high concentration of protein required for this experiment, only low molar amounts were present (4 mg/ml rα2I≈160 μM). In addition to collagens, α2β1 is also known to bind echovirus, laminin and type I collagen C-propeptide (Elices and Hemler, 1989; Bergelson et al., 1992; Weston et al., 1994). Kamata et al. (1994) recently demonstrated that an antibody which blocked α2β1-echovirus binding mapped to the I-domain, suggesting that echovirus may be a ligand for the I-domain. However, preliminary data indicated that rα2I did not bind to laminin or type I collagen C-propeptide in solid-phase binding assays (D. Davies, D. A. Calderwood, D. S. Tuckwell and M. J. Humphries, unpublished data). These ligands may be bound by the non-I-domain region of the integrin, and the exact relationship between the I-domain and the rest of the integrin therefore needs to be established in future studies. There is evidence for synergy between the I-domain and other regions of the integrin: Stanley et al. (1994) recently showed that, for αL, both the I-domain and the N-terminal divalent cation-binding repeats were involved in ICAM-1 binding. Also, the variety of integrin activation states for ligands seen in the non-I-domain integrin α4β1 (Masumoto and Hemler, 1993) are also seen in α2β1. For example, depending on the cell type into which α2 cDNA was transfected, the same cDNA produced α2β1 capable of binding type I collagen and laminin, collagen only or no ligand (Chan and Hemler, 1993), thus demonstrating interactions between I-domain and non-I-domain regions of the integrin. The role of these interactions in the process of collagen binding remains to be established.

Integrin α2 I-domain and the Von Willebrand factor A-domain family

A-domains have to date been identified in types VI, VII, XII and XIV collagens, complement factors B and C2, cartilage matrix protein and integrins α1, α2, αL, αM, αX and αE (αIEL; Colombatti et al., 1993; Shaw et al., 1994), but their functions are still poorly understood. The majority of functional studies have been caried out on von Willebrand factor (vWf) and its three A-domains, A1, A2 and A3. Types I, III and VI collagen have been shown to bind to vWf (Rand et al., 1991; de Groot et al., 1988) and binding sites for types I and III collagen have been localised to A1 and A3 domains (Roth et al., 1986; Pareti et al., 1987; Mohri et al., 1989; Piétu et al., 1989; although see Cruz et al., 1993 and Sixma et al., 1991). Together with the data for rα2I in this report, collagen binding is therefore seen to be a property of a number of A-domains. It is, however, unlikely that collagen binding is a feature common to A-domains: Type XIV collagen may not bind collagens (Brown et al., 1993, although see Just et al., 1991) and the A-domain-containing N-terminal region of α3(VI) collagen did not bind type I or III collagens, although it did bind to the collagenous domain of type VI collagen (Specks et al., 1992). In addition, no collagen ligands have yet been described for αLβ2, αMβ2 or αXβ2 integrins although other ligands have been reported (reviewed by Hynes, 1992; Tuckwell and Humphries, 1993).

A number of studies have attempted to define the function of integrin I-domains using chimeric, truncated or mutated integrin constructs: The αM I-domain was found to be a recognition site for monoclonal antibodies which blocked binding of the intact integrin (αMβ2) to its ligands iC3b, fibrinogen and ICAM-1 (Diamond et al., 1993) and mutagenesis of the αM I-domain abolished iC3b binding (Michishita et al., 1993). In addition, the αL I-domain was found to be the binding site for a monoclonal antibody which could regulate the binding of αLβ2 to ICAM-1 (Landis et al., 1993). Recent studies employing recombinant I-domains have demonstrated αL I-domain binding to ICAM-1 (Randi and Hogg, 1994) and αM I-domain binding to ICAM-1 and fibrinogen (Zhou et al., 1994). It seems very likely that C3b and the homologous protein C4b are also A-domain ligands as, in addition to data for αM I-domain and iC3b, αXβ2 has been reported to bind iC3b (Hynes, 1992; Tuckwell and Humphries, 1993), and complement factors B and C2, which contain I-domains, bind C3b and C4b, respectively (Kinoshita, 1991).

It is now also apparent that the A-domains contain a cation binding site, as divalent cation binding was demonstrated for a recombinant αM I-domain (Michishita et al., 1993) and binding of rα2I to collagen was divalent cation-dependent. A cation-independent component of binding was seen as the concentration of biotinylated I-domain was increased (Fig. 7). However, identical studies using unmodified I-domain did not show cation-independent binding. It is possible that modification of the protein may have partly affected its ability to bind cations.

In conclusion, we have directly demonstrated a collagen-binding function for integrin α2 I-domain, showing that it binds specifically to types I, II and XI collagens in a cation-dependent manner. This will not only clarify our understanding of integrin-ligand binding but will also add to the information on the functions of the A-domain family.

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Note

Submission of this manuscript coincided with the publication of a study by Kamata et al. (*J. Biol. Chem.* (1994) **269**, 26006-26010) demonstrating binding of recombinant I-domain to collagen. Our study is in broad agreement with and extends the work of Kamata et al., with the exception of the observation of Kamata et al. that binding of iodinated I-domain to collagen is cation-independent. Our study shows that reagent concentration and perhaps also protein modification are modulators of cation dependency, and these factors may account for the difference between our data and that of Kamata et al.

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